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MECHANISMS OF HEXOSAMINE-INDUCED CHOLESTEROL ACCUMULATION AND THERAPEUTIC ACTIONS OF CHROMIUM

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Dedication

This dissertation is dedicated to my family. I would not be where I am today without their constant love, support, and guidance. I would like to thank my mother for always encouraging me to strive to my full potential. I would also like to thank my father for being my greatest advocate. They have both provided me with the values and dedication to always persist in all of my endeavors, both academic and personal. I would also like to thank my sister for being a source of advice and willing to listen. I thank my extended family and close peers for their continued prayers and thoughts throughout my academic journey.

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Abstract

Brent A. Penque

MECHANISMS OF HEXOSAMINE-INDUCED CHOLESTEROL ACCUMULATION AND THERAPEUTIC ACTIONS OF CHROMIUM

Excess caloric intake and/or obesity currently remain the largest predisposing risk factors for the development of type 2 diabetes. Discerning the cellular and molecular mechanisms responsible and amendable to therapy represents a growing challenge in medicine. At a cellular level, increased activity of the hexosamine biosynthesis pathway (HBP), a sensor of excess energy status, has been suggested to promote the exacerbation of insulin resistance through increasing adipose tissue and skeletal muscle membrane cholesterol content. This in turn compromises cortical filamentous actin structure necessary for proper incorporation of the insulin-sensitive glucose transporter GLUT4 into the plasma membrane. The current studies attempted to elucidate the mechanism by which hexosamines provoke membrane cholesterol toxicity and insulin resistance. In 3T3-L1 adipocytes cultured with pathophysiologic hyperinsulinemia to induce insulin resistance, increased HBP flux was observed. This occurred concomitant with gains in the mRNA and protein levels of HMG-CoA reductase (HMGR), the rate limiting enzyme in cholesterol synthesis. Mechanistically, immunoprecipitation demonstrated increased HBP-induced *N*-acetylglucosamine (*O*-GlcNAc) modification of specificity protein 1 (Sp1), a regulator of HMGR synthesis. This was associated with increased affinity toward

and activity of *Hmgcr*, the gene encoding HMGR. Global HBP inhibition or Sp1 binding to DNA prevented membrane cholesterol accrual, filamentous actin loss, and glucose transport dysfunction. Furthermore, hyperinsulinemia and HBP activation impaired cholesterol efflux in adipocytes, exacerbating cholesterol toxicity and potentially contributing to cardiovascular disease. In this regard, chromium picolinate (CrPic), known to have beneficial effects on glucose and lipoprotein metabolism, improved cholesterol efflux and restored membrane cholesterol content. To test the role of membrane cholesterol accumulation *in vivo*, studies were conducted on C57Bl/6J mice fed a low or high fat diet. High fat feeding promoted increased HBP activity, membrane cholesterol accumulation, and insulin resistance. Supplementation of mice with CrPic in their drinking water (8µg/kg/day) countered these derangements and improved insulin sensitivity. Together, these data provide mechanistic insight for the role of membrane cholesterol stress in the development of insulin resistance, as well as cardiovascular disease, and highlight a novel therapeutic action of chromium entailing inhibition of the HBP pathway.

Jeffrey S. Elmendorf, Ph.D., Chair

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Abbreviations

2-DG	2-deoxy-D-glucose
ABCA1	ATP-binding cassette transporter 1
ACC	Acetyl-CoA carboxylase
AICAR	5-amino-1-β-D-ribofuranosyl-imidazole-4-carboximide
AMP	5' adenosine monophosphate
AMPK	5' AMP-activated protein kinase
aPKC	Atypical protein kinase C
ApoA-I	Apolipoprotein I
Arp2/3	Actin-related proteins 2/3
AS160	Akt substrate of 160 kilodaltons
Atf6	Activating transcription factor 6
ATP	5' adenosine triphosphate
AUC	Area under the curve
C/EBP	ccaat-enhancer binding protein
CAD	Coronary artery disease
CaMKKβ	Calmodulin-dependent protein kinase β
cAMP	cyclic AMP
CBS	Cystathionine-β-synthase
ChIP	Chromatin immunoprecipitation
Chop	C/EBP homology protein
Chrebp	Carbohydrate response element binding protein
CNS	Central nervous system
COPII	Coat protein complex II

Cr^{3+}	Trivalent chromium
Cr^{6+}	Hexavalent chromium
CrCIT	Chromium citrate
$CrCl_3$	Chromium chloride
CREB	cAMP response element binding
CrN	Chromium nicotinate/niacin
CrPic	Chromium picolinate
CRTC2	CREBP regulated transcription coactivator 2
CrY	Chromium yeast
CVD	Cardiovascular disease
DMEM	Dulbecco's Modified Eagle Medium
DON	6-diazo-5-oxo-L-norleucine
EM	Endosomal membrane
ER	Endoplasmic reticulum
FA	Fatty acid
F-actin	Filamentous actin
FOXO1	Forkhead box protein O1
G6Pase	Glucose-6-phosphatase
GDI	Guanine nucleotide dissociation inhibitor
GFAT	Glutamine:fructose-6-phosphate amidotransferase
GlcN-6-P	Glucosamine-6-phosphate
GLP1	Glucagon-like peptide 1
GLUT	Glucose transporter
GSV	Glucose storage vesicle

HBP	Hexosamine biosynthesis pathway
HDL	High density lipoprotein
HF	High fat
HMG-CoA	3-hydroxymethyl-3-glutaryl coenzyme A
HMGR	HMG-CoA reductase
IGT	Impaired glucose transport
IL-6	Interleukin 6
Insig	Insulin inducible gene
IPGTT	Intraperitoneal glucose tolerance test
IPITT	Intraperitoneal insulin tolerance test
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
KIF3	Kinesin family member 3
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
LF	Low fat
LKB1	Liver kinase B1
LMWCr	Low-molecular-weight chromium binding substance
LXR	Liver X receptor
MTR	Mithramycin
NPC	Niemann-Pick Disease, Type C
OGA	O-GlcNAcase
OGT	O-GlcNAc transferase
PBS	Phosphate buffered saline

PDK1	Phosphoinositide-dependent kinase 1
PEPCK	Phosphoenolpyruvate carboxykinase
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator-1α
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKB	Protein kinase B
PLD	Phospholipase D
PM	Plasma membrane
RCT	Reverse cholesterol transport
ROS	Reactive oxygen species
RXR	Retinoic X receptor
SCAP	SREBP cleavage-activating protein
SEM	Standard error of the mean
siRNA	Small interfering RNA
SNARE	Soluble NSF attachment protein receptor
Sp1	Specificity protein 1
SRE	Sterol response element
SREBP	Sterol regulatory element binding protein
STAT3	Signal transducer and activator of transcription 3
Stp	Streptavidin
T2D	Type 2 diabetes
TGN	Trans-Golgi network
TLR4	Toll-like receptor 4

TPN	Total parenteral nutrition
tSNARE	Target SNARE
TZD	Thiazolidinediones
UDP	Uridine diphosphate
UDP-GlcNAc	UDP-N-acetylglucosamine
VAMP2	Vesicle-associated membrane protein 2
vSNARE	Vesicle SNARE
VSV	Vesicular stomatitis virus
Xbp1	X-box binding protein 1

Chapter I. Introduction

In the health care field, a growing concern has emerged over the increased incidence of chronic disease in patient populations. Complex diseases involving multisystem pathologies are becoming increasingly prevalent. Despite enormous medical progress, type 2 diabetes (T2D), cardiovascular disease (CVD) and cancer remain worldwide health problems. In this regard, excessive caloric intake and sedentary lifestyles in the developed world have greatly contributed to the increased prevalence of T2D. Compounding this “energy balance” model is the fact that rather than a restabilization or plateauing of weight at a higher level, weight continues to escalate, suggesting a major contributing role of dietary composition on metabolic derangements and T2D progression (1). In addition, T2D is known to be an independent risk factor for the development of CVD (2, 3). According to the United States Centers for Disease Control and Prevention 2011 National Diabetes Fact Sheet (4), an estimated 25.8 and 79 million Americans have diabetes and pre-diabetes, respectively. Worldwide projections of the incidence of diabetes continue to escalate each year, with recent predictions that almost a half billion individuals will have T2D by 2030 (5). Augmenting this problem are the healthcare and economic burdens of this disease. In 2007, the total direct and indirect costs associated with T2D were $174 billion. Diabetes affects multiple physiological systems and is the leading cause of blindness, heart failure, stroke, kidney failure, non-traumatic lower limb amputations, and gum disease. The burden this disease continues to have on families necessitates the need for better prevention and treatment. Clinical measures used to delay the onset of this disease, however, are often limited. Diet and exercise are proven,

effective methods to attenuate disease progression, but have been met with limited patient compliance. Efficacious medications have been developed to alleviate insulin resistance, yet reports of adverse effects (e.g., hypoglycemia, weight gain, abnormal liver function, gastrointestinal, lactic acidosis, etc.) compel the need for future research into novel therapies.

Insulin resistance represents a hallmark feature that presents early in the development of T2D and is a major therapeutic target. Insulin resistance clinically is defined as a reduced ability of insulin to lower plasma glucose levels. In skeletal muscle and adipose tissue, this correlates to a loss in insulin-stimulated glucose uptake. In the liver, this results in attenuation of insulin's inhibitory effects on hepatic glucose production and output. Beyond regulating glucose transport, insulin resistance also promotes release of fatty acids (FAs) stored in fat. In a healthy individual, insulin inhibits lipolysis in adipose tissue through the phosphodiesterase 3B-mediated degradation of cellular cyclic AMP (6). This event suppresses protein kinase A-mediated phosphorylation and activation of hormone-sensitive lipase and perilipin A. Inhibition of these proteins prevents the activation of lipases responsible for the breakdown and release of FAs. A primary consequence of insulin resistance is that adipose tissue becomes refractory to the inhibitory effect of insulin, resulting in excess circulating FAs. In addition, insulin resistance and subsequent glucose intolerance itself are widely believed to promote compensatory hyperinsulinemia. This hyperinsulinemia is initially thought to help maintain normoglycemia. Yet hyperinsulinemia, excess circulating FAs, and hyperglycemia have been shown to impinge upon insulin sensitivity *in vitro* as well as in animal and man (7-15). This results in a vicious

cycle promoting further hyperinsulinemia which, in turn, exacerbates insulin resistance. Ultimately, this glucose and lipid toxicity is known to promote the progressive impairment in the ability of the β cells of the pancreas to produce and secrete insulin, leading to the diagnosis of frank T2D.

As insulin resistance represents an early abnormality in the progression of diabetes, research efforts have sought to understand the mechanisms fueling its development. In adipocytes and skeletal muscle, pathophysiologic hyperinsulinemia has been shown to promote insulin resistance in absence of insulin signaling defects (8, 12, 16-18). An emerging appreciation is that downstream defects that transpire at or near the plasma membrane (PM) may contribute to dysregulation of glucose transport into these tissues. In this context, our lab has demonstrated that increases in PM cholesterol content, induced by hyperinsulinemia, perturb cortical filamentous actin (F-actin) structure that is essential for insulin-stimulated glucose transport. Our studies further suggest increased hexosamine biosynthesis pathway (HBP) activity as a basis for hyperinsulinemia-induced increases in PM cholesterol content (7, 11). Interestingly, we have also found increased HBP activity increases endosomal membrane cholesterol levels in adipocytes, impairing the proper trafficking of the ATP-binding cassette transporter (ABCA1), necessary for cholesterol efflux (19).

Therapeutically, the trivalent micronutrient, chromium (Cr^{3+}) has been known since the 1950's to benefit glucose and lipid metabolism (20), yet the mechanism of action remains incompletely understood. Recent study has shown, however, that Cr^{3+} can lower PM and endosomal membrane cholesterol levels, improving insulin sensitivity and cholesterol efflux processes (19, 21, 22). In addition, a

systematic review has also demonstrated that Cr^{3+} effectively improved hyperglycemia in patients with T2D (23).

In the following subsections of this chapter, I will highlight the fundamental actions of insulin in maintaining glucose homeostasis. I will subsequently discuss how this process goes awry in the context of the diabetic milieu, with emphasis on the role of the HBP in promotion of this phenotype. I will then detail mechanisms by which increased HBP activity may impair lipoprotein metabolism, and finally discuss the beneficial actions of Cr^{3+} supplementation on improving glucose and lipid homeostasis. In Chapter II, I will present my thesis work published in Molecular Endocrinology (24) and Arteriosclerosis, Thrombosis and Vascular Biology (19) demonstrating a novel mechanism by which HBP-induced transcriptional activation of cholesterol synthesis impairs glucose and cholesterol transport. I will further present study in preparation for submission to Diabetes demonstrating beneficial actions of Cr^{3+} entail countering aberrant HBP activity and cholesterol accumulation. In Chapter III, I will place my thesis work within the context of the current body of knowledge in the field of diabetes and cardiovascular health. I will also identify future questions and studies which could serve to enhance the understanding of these mechanisms fueling disease progression.

I.A. Insulin-Mediated Glucose Regulation

The hormone insulin is essential for several processes within the body, from regulation of protein synthesis to the modulation of the activity of numerous enzymes. Importantly, insulin is a primary hormone responsible for the regulation

of glucose homeostasis in the post-prandial state. Upon ingestion of a meal, glucose enters the bloodstream and is sensed by the endocrine pancreas, specifically β cells in the islets of Langerhans. Glucose then stimulates the secretion of insulin from the pancreas into the circulation. Importantly, insulin is a relatively fast-acting hormone that needs to respond to rapidly changing levels of glucose in the bloodstream. As such, the half-life of insulin in the plasma is actually quite short, ranging from 4-6 minutes (25, 26). In fact, at first pass to the liver, approximately 50% of insulin is cleared from the body through receptor-mediated endocytosis. Insulin that has not been cleared exerts its primary effects on glucose regulation in hepatic and adipose tissue, as well as skeletal and cardiac muscle. In the liver, insulin decreases gluconeogenesis and glycogenolysis while stimulating glucose storage as glycogen. In striated muscles and adipose tissue, insulin stimulates glucose transport, allowing for glucose flux through oxidative pathways to produce the energy needs of the cell. Remaining systemic insulin is ultimately removed by endocytic processes in insulin-sensitive tissues or by reabsorption of insulin in the proximal tubular cells of the kidney. In addition, insulin also negatively impinges upon its own secretion in the β cell. Through these processes, the body is able to constantly respond and precisely control whole-body glucose homeostasis. Under healthy conditions, plasma glucose concentrations will not fall below 70 mg/dL or increase beyond 100 mg/dL in the fasted state. Further, two hours following an oral glucose tolerance test, plasma glucose should not rise above 140 mg/dL. The focus of this thesis is to understand the molecular mechanisms of insulin-mediated glucose transport

into adipose tissue and skeletal muscle and how this process becomes dysregulated into the context of insulin resistance.

I.A.1. Integrative Actions of Insulin

At a cellular level, insulin-stimulated glucose transport into adipose tissue and striated muscle is mediated through its ability to stimulate the subcellular trafficking of the insulin-sensitive glucose transporter GLUT4 from intracellular pools to the PM (27-29). While at least 14 other glucose transporter isoforms exist in various tissues (30), GLUT4 is localized to insulin-sensitive striated muscle as well as brown and white adipose tissue. The binding of insulin to its surface receptor on the PM triggers a large increase in the exocytosis of GLUT4 to the PM. In adipocytes and L6 muscle cells, insulin has also been shown to reduce the rate of GLUT4 endocytosis (31-34). This rate-limiting step in glucose uptake and utilization results in the population of the PM with GLUT4 to facilitate glucose entry into these tissues. In fact, approximately 90% of post-prandial glucose disposal is mediated in these two tissues (35).

Extending beyond striated muscle and adipose tissue, the liver plays a major role in the maintenance of glucose homeostasis. Insulin exerts its effects on the liver through post-translational modification and transcriptional repression of genes involved in glucose production (36). Upon binding to its cell surface receptor in hepatocytes, insulin stimulates a phosphorylation cascade that results in the phosphorylation of Akt (protein kinase B, PKB) which in turn phosphorylates the forkhead box protein (FOXO1). This phosphorylation event has been shown to restrict the access of FOXO1 to the nucleus, thereby

suppressing its transcription of glucose-6-phosphatase (G6Pase) as well as phosphoenolpyruvate carboxykinase (PEPCK), two enzymes involved in gluconeogenesis (37). By modulation of FOXO1 activity, insulin is also able to suppress peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), a transcriptional co-activator known to activate the entire gluconeogenic pathway (38). In effect, this modification of FOXO1 prevents its association with PGC-1α to activate gluconeogenesis. Gluconeogenesis is also indirectly suppressed in the liver through a decrease in the secretion of glucagon by the α cells of the endocrine pancreas.

Finally, recent studies have provided evidence that the central nervous system (CNS) also plays a significant role in glucose homeostasis (39-43). CNS regulation of food intake is a complex process involving a variety of short-term and long-term signals. Yet insulin action on the CNS is now thought to mediate glucose homeostasis through inhibition of hepatic glucose output. For example, insulin is known to activate ATP-sensitive potassium channels in neurons found in the arcuate nuclei of the hypothalamus (44, 45). Activation of these channels through central administration of diazoxide promotes a reduction in blood glucose levels in rats (46). While the precise mechanisms by which CNS insulin action may inhibit hepatic glucose production are unknown, it is suggested that it requires interleukin 6-mediated phosphorylation of hepatic signal transducer and activator of transcription 3 (STAT3) (41, 42, 47). Phosphorylation of STAT3 reduces PEPCK and G6Pase expression and protein levels, thereby inhibiting gluconeogenesis. Despite this, other study suggests that insulin's direct inhibition on hepatic glucose output is dominant, while insulin's effects on the CNS to

inhibit this process are redundant (48). These data suggest that dysregulated gluconeogenesis in insulin-resistant states may also be due to a loss of insulin action in the CNS. Regardless of the exact role of insulin action in the CNS, insulin resistance in the brain is known to contribute to cognitive impairment and Alzheimer's disease, recently characterized as a potential "type 3 diabetes" (49).

I.A.2. Insulin Signaling and GLUT4 Recruitment

The precise networks by which insulin regulates glucose transport via recruitment of GLUT4 to the PM in fat and muscle cells are complex and still imperfectly understood. Upon binding of insulin to the PM localized insulin receptor, the intrinsic tyrosine kinase activity of the receptor is stimulated (**Fig. 1**). This event results in the autophosphorylation of tyrosine residues on the β-subunit (50, 51). Phosphorylation of the insulin receptor stimulates a phosphorylation cascade involving the insulin receptor substrate (IRS) docking proteins. Although there are 6 different isoforms of IRS, IRS1 and IRS2 have been established through knockdown studies in cell culture and mice to be the primary mediators of glucose transport (52-54).

The next step leading to translocation of glucose storage vesicles (GSVs) containing GLUT4 to the PM requires the activation of phosphatidylinosital 3-kinase (PI3K) by IRS. PI3K stimulates the conversion of phosphatidylinositol 4,5-bisphophate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3). PIP3 then facilitates the recruitment Akt and phosphoinositide-dependent kinase 1 (PDK1) to the PM, where Akt then becomes phosphorylated and active. Akt subsequently phosphorylates and inhibits the GTPase activating domain (GAP) of Akt

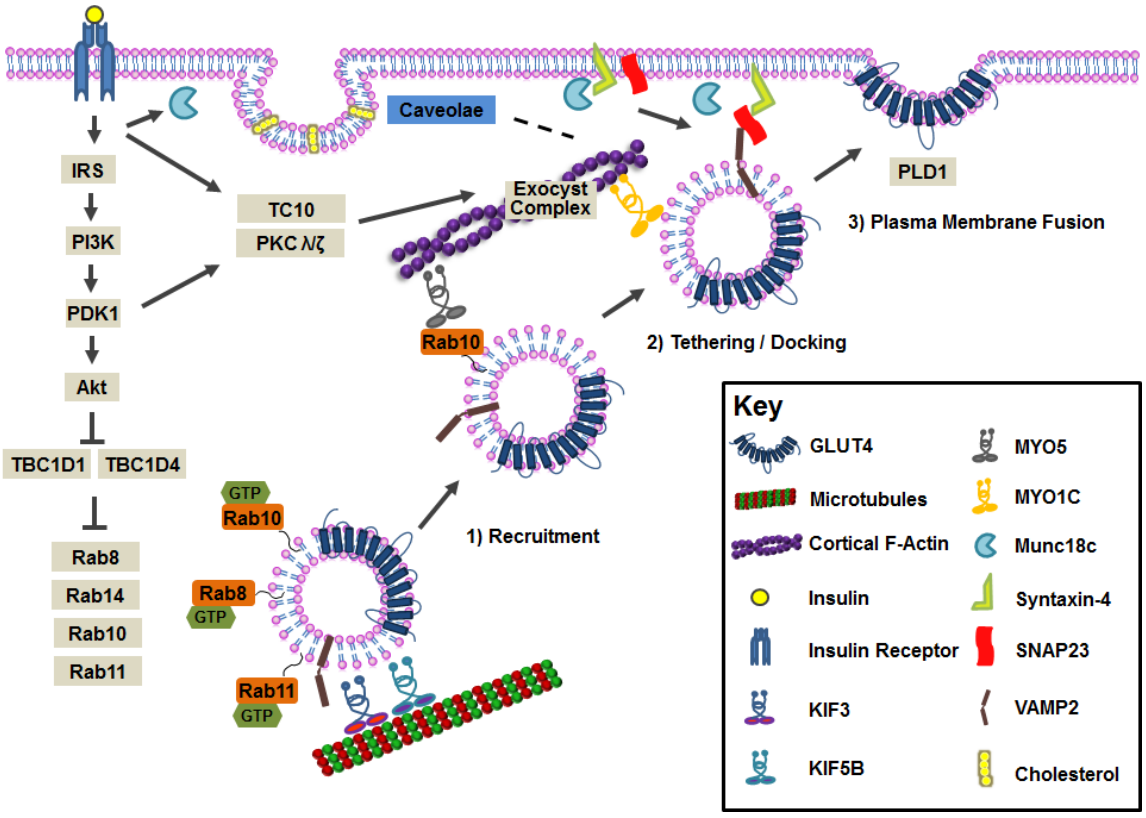

Figure 1. Schematic of the steps involved in GLUT4 recruitment, tethering and docking, and fusion.

Insulin binding to its receptor triggers a phosphorylation cascade resulting in the activation of tissue specific Rab proteins. These Rab proteins are in turn responsible for the recruitment of GLUT4 containing vesicles along microtubule and cytoskeletal tracks to the PM. A host of signaling, motor, cytoskeletal, and membrane proteins and lipids play vital roles in this trafficking process (see text for expanded details).

substrate of 160-kilodaltons (AS160, TBC1D1). Currently, there are three known isoforms of Akt. Similar knockdown studies identified Akt2 as the predominant isoform responsible for glucose uptake into muscle and fat tissues, although Akt1 is thought to play a minor role (55-57). Phosphorylation of AS160 allows for Rab-mediated trafficking of GSVs to the PM. Of note, another Rab-GAP, TCB1D1, has been implicated in insulin-mediated regulation of GLUT4 exocytosis and is particularly abundant in skeletal muscle (58, 59). The tissue-specific isoforms of Rab proteins targeted by AS160 and TCB1D1 are currently under study. Rab8A and Rab14 are postulated to be the predominant isoforms in skeletal muscle, whereas Rab10 is the important isoform in adipose tissue (60). Additionally, Rab4, 5, and 11 have also been shown to associate with the GSVs (61, 62).

The subsequent steps by which Rab proteins mediate the translocation of GSVs are under investigation. Two models have been proposed to explain how insulin regulates these distal effects. The most popular model is the retention model (63). In this model, GSVs are anchored to the interior of the cell, preventing movement on cytoskeletal tracks under basal conditions. In the absence of insulin stimulation, Rab proteins are found in inactive states and thus hinder the vesicle attachment to the microtubules. Support for this hypothesis comes from research that has shown Rab4 binding to the microtubule motor protein kinesin family member 3 (KIF3) is dependent on insulin (64, 65). Another model, the repulsion model, suggests that GSVs can constantly access the PM, despite the absence of insulin (63). Rather, GSVs that arrive at the PM are repelled, and the action of insulin is to stimulate fusion and prevent this repulsion. Evidence for this model comes from recent advances in the field of microscopy

that allow for the resolution of GLUT4 within 150 nm of the PM. Total internal reflection fluorescence microscopy supports this contention as numerous studies have shown these structures near the membrane in the absence of insulin (66-68). A complete understanding of the precise mechanisms leading to GLUT4 docking and fusion at the PM will require higher resolution techniques to examine these processes in live cells.

In addition to the canonical insulin signaling pathway, phosphoinositides derived from PI3K are known to activate atypical protein kinase C (aPKC, PKCλ/ζ). Knockout of PKCλ specifically in mouse muscle provokes systemic insulin resistance and glucose intolerance (69), suggesting an important role in mediating insulin-stimulated glucose uptake. It is believed that aPKC acts at the level of promoting GLUT4 translocation, and studies suggest that PKCλ and PKCζ may orchestrate these processes through Rac1-mediated reorganization of the cytoskeleton (70).

I.A.3. GLUT4 Trafficking and PM Fusion

Conceptually, it is evident that GLUT4 trafficking to the PM requires the coordinated effort of multiple carriers along its track. In the basal state, GLUT4 can be found in both GSVs as well as in larger structures formed from the trans-Golgi network (TGN) and endosomes. In adipocytes, movement of GLUT4 along microtubule tracks is thought to be mediated primarily by kinesin superfamily proteins KIF5B and KIF3 (64, 65). As mentioned previously, in rat adipose cells, cryosectioning and immunoelectron microscopy showed that GLUT4 was located just beneath the PM (71). In line with the repulsion model, this suggests that the

major function of these microtubule networks is to continuously supply GLUT4 to the PM.

Having reached close proximity to the PM via the microtubule network, several complexes have been suggested to serve independently or in collaboration to tether the vesicle to the PM for fusion. It is well documented that F-actin just below the surface of the membrane plays a role in this process (72-74). Functionally, insulin causes a rapid remodeling of actin into a cortical mesh required for GLUT4 translocation. Study has identified myosin motors MYO5 and MYO1C on GSVs and suggested these may serve to anchor them to the actin meshwork (75-77). Essential to the rearrangement of the actin to facilitate this anchoring are several insulin-regulated proteins (e.g., TC10 (78, 79)), actin-related proteins 2/3 (Arp2/3), and Cofilin, Myo1c, Rac1, and focal adhesion kinase (80). Additionally, phosphoinositides are also known to regulate the actin cytoskeleton necessary for GLUT4 translocation (72). Multiple studies have shown that stimulating the hydrolysis or sequestering PIP2 results in a depletion of cortical F-actin (81-85). Importantly, the precise regulation of these processes leading to the formation of this F-actin mesh is necessary for GLUT4 arrival at the PM. Disruption of the F-actin structure using the actin depolymerizing agent cytochalasin D, for instance, causes an inhibition in insulin-stimulated GLUT4 redistribution (86-88).

In addition to the actin meshwork, the formation of what is referred to as the exocyst, perhaps mediated by actin itself, is needed for the arrival of GLUT4 to the PM. This octameric protein is responsible for the spatial targeting of GSVs to the PM. Research has shown that the exocyst has numerous interactions with

phospholipids at the PM, actin, Arp2/3, aPKC, small G-proteins (Rab8, Rab10, Rab11), and soluble NSF attachment protein receptors (SNARE) (89-91). The formation of the exocyst complex upon insulin stimulation is thought to occur at PM caveolae regions, small invaginations (50-100 nm) in the PM rich in lipids and cholesterol (80). Binding of GSVs to the exocyst complex ultimately leads to the formation of a SNARE complex responsible for GLUT4 fusion with the PM.

The SNARE complex consists of both target membrane SNARES (tSNARES), SNAP-23 and Syntaxin-4, as well as vesicle SNARE (vSNARE) protein VAMP2. The association of these vesicle and target membrane SNARES is thought to promote the final steps in the fusion process. This process has been described as analogous to the closing of a zipper. The formation of a complex between tSNARES and vSNARES is highly stable. The stability conferred by this binding and subsequent “zippering” is thought to overcome the energy barrier required, thereby serving as a catalyst to merge these two membranes (92). An important regulator of this process is the protein Munc18c, which itself is activated by the insulin receptor (93, 94). Munc18c is known to be a direct binding partner of Syntaxin 4 (93). One hypothesis suggests that Munc18c is normally inhibitory to GLUT4 exocytosis by keeping Syntaxin 4 in an inactive conformation. Phosphorylation of Munc18c by the insulin receptor then results in its dissociation from Syntaxin 4, allowing for complex formation to occur. Support for this comes from studies showing that adipocytes from Munc18c null mice have an increased sensitivity to insulin-stimulated GLUT4 translocation (95). Additionally, overexpression of Munc18c inhibits GLUT4 exocytosis (5, 96-98). Recent study has also suggested that insulin signaling through conventional PKC

or PKCζ may promote the dissociation between these two proteins (99, 100). Future studies are aimed at carefully dissecting how this phosphorylation event precisely directs the final mechanisms leading to PM fusion events.

I.A.4. PM Lipids Regulating GLUT4 Exocytosis

As previously mentioned, the formation of the complex machinery that facilitates GLUT4 translocation is thought to occur just below the PM in caveolae invaginations, stabilized by the caveolin proteins (101). Although the investigation of these structures has proven extremely difficult, double labeling studies have shown that F-actin appears to emanate from the neck regions of caveolae (87, 102). In addition, disruption of the caveolae with the cholesterol solubilizing agent, methyl-β-cyclodextrin, was shown to disrupt the localization of this F-actin (87). These findings suggest that this lipid-rich region of the PM may play an essential role in mediating F-actin reorganization necessary for optimal tethering, docking, and fusion events. Further support for this comes from findings that in adipocytes, these lipid regions are necessary for the activation of TC10 (103, 104). A key role of PIP2 has been proposed in this process as electron microscopy has shown high concentrations of this lipid at the rim of caveolae structures, consistent with its role in regulating the actin cytoskeleton (105). These findings are interesting in the context of work in our lab suggesting that hyperinsulinemia promotes a loss of PIP2 and F-actin structure and that exogenous add back of PIP2 restores F-actin structure and insulin responsiveness (8, 12). Of further note, hyperinsulinemia and hyperlipidemia have been shown to provoke increases in PM cholesterol content correlated with

these losses in PIP2 and F-actin in 3T3-L1 adipocytes and L6 myotubes. Strikingly, removal of this excess cholesterol reversed a loss in F-actin structure and impaired insulin responsiveness (11). Together, this data formed a basis for my thesis work aimed on dissecting how metabolic syndrome promotes cholesterol accumulation at the PM.

Another role of lipids in regulating the final steps in fusion events comes from studies regarding phospholipase D (PLD) activity. PLD has been shown to be required for the insertion of GLUT4 into the PM (106, 107). Insulin stimulation in adipocytes is known to recruit PLD to the PM. While the exact mechanisms of a direct role of PLD in mediating fusion have yet to be elucidated, PLD is known to activate phosphatidylinositol 4-phosphate 5-kinase, resulting in the production of PIP2 necessary for exocytosis (108, 109). It is also believed that PLD may serve a function in lowering the energy of activation needed for membrane curving during the generation and expansion of fusion pores (110).

In summary, a wide variety of machinery is responsible for signaling GLUT4 exocytosis. The precise molecular details regarding the final steps are continuing to emerge. The role of my current work was to gain further insight into how these processes may go awry in the context of the diabetic milieu. Importantly, my research has focused on methods to correct against abnormalities in these processes to attempt to restore insulin responsiveness. Finally, alterations in these regulatory nodes have been shown to decrease the generation of atheroprotective high density lipoprotein cholesterol (HDL). Thus, attempts to establish new strategies to attenuate insulin resistance and alterations in lipoprotein metabolism are of pivotal importance.

I.B. Mechanisms of Insulin Resistance

Over the past 20 years, the major driving force for research in the diabetes field was the attempt to determine the chief molecular derangement responsible for the development of insulin resistance. Since then, it is recognized that an increasing number of cellular, endocrine, inflammatory, and neuronal derangements are involved in this process (111). A major clinical challenge is that while many of these pathways are interdependent, therapeutic treatments that counter just one mechanism may fail to result in significant glycemic improvements in patients. In this regard, many of the same cellular derangements (ER, oxidative, PM stress, and mitochondrial dysfunction) occur in multiple tissues, creating further challenges for therapeutic intervention. Thus, a concerted effort to discern the precise temporal sequence for appearance of these effects may prove invaluable to prevent the exacerbation of insulin resistance to β cell failure. The goal of this section is to review the primary mechanisms involved in fat, glucose, and insulin-induced insulin resistance, with emphasis on the emerging role of the HBP in fueling many of these processes. This section will also highlight how insulin resistance may promote alterations in lipoprotein metabolism, a secondary goal of my research.

I.B.1. Fat-Induced Insulin Resistance

It has been widely recognized that plasma concentrations of FAs are commonly elevated in obese, insulin resistant humans and that this process is mainly driven by an inappropriate storage/release of FA stores in adipocytes (112-114). It should be noted, however, that clinical trials have shown that

elevations in intake of FAs can impair insulin sensitivity independent of body weight (115). The first model whereby FAs could disrupt glucose metabolism came in 1963 when Philip Randle postulated that a competition could occur between oxidation and utilization of FAs or glucose in insulin-sensitive tissues (116). This model, referred to as the Randle cycle, involves glucose oxidation's generation of malonyl-CoA which can bind to and inhibit tissue-specific isoforms of carnitine palmitoyltransferase. This binding prevents FA transport to the mitochondria, thereby suppressing FA oxidation. In the years following, greater attention has been on the role FAs could have on suppressing glucose uptake rather than oxidative pathways. This led to the formation of the Shulman hypothesis, which suggests that FA metabolites, such as ceramides, diacylglyerol, and acyl-CoA, play active roles in inhibiting insulin-stimulated glucose transport (117). These signaling proteins can activate c-Jun N-terminal kinases (JNK), either through activation of PKCθ or through toll-like receptor 4 (TLR4), leading to attenuation of insulin signaling by inhibitory Ser/Thr phosphorylation and/or degradation of the insulin receptor (111, 118-120).

An additional observation with regard to FA-induced insulin resistance is that insulin resistance is dictated not just by the amount, but also by the specific composition of FAs ingested (115). For instance, studies in animal and man demonstrate that HF diets rich in saturated FAs impair insulin action (52, 121-123). One particular randomized crossover study suggests that in healthy individuals, saturated FAs may not impair insulin sensitivity, but rather that overweight individuals may be more susceptible to insulin resistance due to saturated FAs (124). This may suggest that saturated FAs are an exacerbating

factor in the development of insulin resistance. Nevertheless, the saturated FA palmitate, most prevalent in the circulation, was found to induce insulin resistance through *de novo* synthesis of ceramides (125). These studies have shown that this process entails palmitate increasing mRNA encoding dihydroceramide desaturase 1, which then converts dihydroceramide to ceramide (126). Increases in ceramides, in addition to previously discussed activation of JNK, have also been shown to result in decreases in the level of Akt phosphorylation (11, 127, 128). Mechanistically, study has suggested this is due to an impairment in insulin's ability to translocate Akt to the PM mediated through PKCζ phosphorylation of Akt on its PH domain (129). Other research suggests that ceramides activate protein phosphatase 2A, leading to removal of phosphorylation of Akt as well as inhibiting PKCλ (130, 131). Importantly, study has demonstrated that saturated FA treatment of muscle cells promotes macrophage activation, further impinging upon insulin sensitivity (132).

Recent work in our laboratory has suggested a new role of palmitate-induced insulin resistance entailing increased glucose flux through the HBP (discussed below) in mediating alterations in PM architecture necessary for glucose transport into skeletal muscle (11). In these studies, palmitate treatment was found to also promote the characteristic reduction in insulin-stimulated phosphorylation of Akt2 and AS160 (11, 127). However, removal of excess PM cholesterol using methyl-β-cyclodextrin restored insulin responsiveness despite attenuation of insulin signaling at the level of these two key proteins. Interestingly, RL2 labeling showed an increase in HBP activity in palmitate treated cells that was reversed with HBP inhibition. This is consistent with other

reports showing that lipid infusion in rats or palmitate treatment in myotubes promotes increased flux through this pathway (133, 134). This increase in HBP activity was associated with gains in PM cholesterol content, loss in F-actin staining, as well as impaired glucose transport. Strikingly, these PM defects were found to be reversed upon HBP inhibition, promoting appropriate glucose transport (11). Together, these studies suggest that an important component of fat-induced insulin resistance may entail distal signaling derangements that, if corrected, could afford protection against insulin resistance.

I.B.2. Glucose and Insulin-induced Insulin Resistance

Similar to FAs, increases in circulating levels of glucose and insulin are known contributors, both *in vitro* and *in vivo*, to the exacerbation of insulin resistance (8, 9, 12-15, 19, 22, 135-139). As mentioned previously, the production of insulin resistance is thought to result in compensatory hyperinsulinemia, thereby attenuating hyperglycemia. This line of thought, however, implies a primary defect that must initially be responsible for hyperinsulinemia itself. Thus, the temporal sequence of events leading to pathology is still being elucidated. A review by the late Dennis McGarry suggests that perhaps hyperinsulinemia is the primary defect, thus promoting insulin insensitivity and hyperglycemia (140). In fact, the idea of insulin-induced insulin resistance in humans is not a recent phenomenon. As early as 1938, Somogyi reported that patients who were switched from receiving high doses of insulin to low doses had improved insulin sensitivity (141). While complications of high dose insulin treatment were initially attributed as bouts of hypoglycemia leading

to a rebound hyperglycemia (termed the Somogyi effect), new studies exclude this possibility (142) and strongly suggest insulin-induced insulin resistance as the mechanism (143, 144).

Numerous population-based studies have attempted to discern exactly what cutoff should be used to account for hyperinsulinemia in man. In the San Antonio Heart Study, fasting and 2 h post-glucose plasma insulin concentrations were measured in ~3000 individuals with various insulin sensitivities. In this study, hyperinsulinemia was defined in those individuals whose 2 h post-glucose plasma insulin concentrations were 2 standard deviations above the mean of the non-obese, non-diabetic individuals (135). These levels ranged between 31-700 μU/ml (186 pM - 4.2 nM). Strikingly, the prevalence of obesity, T2D, hypertension, and hypertriglyceridemia were found to be two to three times more prevalent in individuals who had insulin levels above 31 μU/ml (186 pM), the low cutoff for hyperinsulinemia (145). Mechanistically, cell culture studies have clearly demonstrated that chronic exposure to supraphysiological (≥100 nM) insulin concentrations induces alterations in the expression and/or activity levels of insulin signaling proteins. However, several groups (13, 146), including our own (7, 8, 12), have shown that 12-14 hour exposure to more physiologically relevant insulin concentrations (500-5000 pM) induces insulin resistance despite intact insulin signaling. Rather, this suggests that distal mechanisms in the insulin signaling pathway may also become impaired, perhaps at earlier stages in disease progression, contributing to the exacerbation of insulin resistance. Within that context, work from our laboratory postulates a mechanism involving excessive glucose flux through the HBP in provoking PM cholesterol

accumulation and a loss in PIP2 regulation of cortical F-actin. The role of the HBP in mediating these processes will be the next topic of discussion.

I.B.3. Hexosamine Biosynthesis and Insulin Resistance

Dating to the 1980's, it was first discovered that the HBP could modify serine/threonine residues of proteins through the addition of the hexosamine β-D-N-acetyl-glucosamine (147, 148). This highly dynamic modification was found to be particularly enriched on transcription factors (149), and coupled to its regulation by insulin, excess nutrient bioavailability, and stress, was proposed to be a sensor of nutrient overload in the cell (150). Marshall et al. first pioneered studies in adipocytes demonstrating that increased routing of glucose through the HBP contributed to desensitization of the glucose transport system (151). This insight came about serendipitously based on other study that demonstrated that treatment of adipocytes with glucose and insulin failed to induce insulin resistance when the cells were cultured in a Hepes-buffered salt solution. It was identified that the amino acid L-glutamine, a substrate for the rate limiting enzyme in the HBP, glutamine:fructose-6-phosphate amidotransferase (GFAT), was necessary for modulating the loss of maximal insulin responsiveness (152). The first step in glucose entry into the HBP is catalyzed by GFAT, which converts fructose-6-phosphate into glucosamine-6-phosphate (GlcN-6-P). GlcN-6-P is subsequently metabolized culminating in the production of UDP-N-acetylglucosamine (UDP-GlcNAc), the high energy substrate of *O*-GlcNAc transferase (OGT), a nuclear and cytosolic enzyme that catalyzes the addition of GlcNAc to serine/threonine residues by *O*-linkage (153, 154). It should be noted

that under healthy conditions, the HBP is a relatively minor pathway in the utilization of glucose, accounting for only 2-5% of glucose flux.

Since the initial discovery that HBP could result in an impairment in insulin responsiveness in murine adipocytes, numerous studies have attempted to discern the precise role of the HBP in this process. Several subsequent cell culture and animal models have clearly demonstrated a role of this pathway in the development of insulin resistance (155-159). For instance, overexpression of GFAT in liver, muscle, or fat has been demonstrated to impair glucose disposal (156, 158, 160, 161). Furthermore, increased HBP activity has been shown to promote hyperleptinemia (162), and glucosamine infusion in rats increased expression of leptin and resistin, particularly in visceral adipose deposits (163). In addition to animal models, polymorphisms in O-GlcNAcase (OGA), the enzyme that hydrolyzes *O*-GlcNAc moieties, have been shown to increase the risk of T2D in the Mexican American population (164). In addition, skeletal muscle biopsies from T2D patients revealed increases in the mRNA and protein levels of GFAT (134, 165). While a large body of research demonstrates HBP-induced insulin resistance, the exact mechanisms are incompletely understood.

I.B.4. Hexosamines and the Liver

Several studies have provided mechanistic insight into how increased HBP activity could promote or exacerbate the insulin resistant phenotype. In the liver, in addition to inhibiting gluconeogenesis, insulin also stimulates FA synthesis. In insulin-resistant states, selective insulin resistance occurs in which insulin fails to signal inhibitory effects on gluconeogenesis while signaling to FA synthesis

remains unscathed (36). This, coupled with hyperinsulinemia, can lead to dysregulated FA production which can further exacerbate insulin sensitivity. With regard to the HBP, study has shown that FOXO1 overexpression reduces the activity of the carbohydrate response element binding protein (Chrebp), a key regulator of lipogenesis, and that it mediates this process through suppressing *O*-linked glycosylation and reducing Chrebp stability (166). In addition, mass spectroscopy and subsequent co-immunoprecipitation has revealed PGC-1α is modified by *O*-GlcNAc and that PGC-1α specifically targets OGT to FOXO1, mediating its subsequent glycosylation (167). Increased *O*-GlcNAc of FOXO1 by hyperglycemia is, somewhat paradoxically, well established to increase its activity toward gluconeogenesis (168). Furthermore, cAMP response element-binding (CREB) regulated transcription coactivator 2 (CRTC2) also is modified by and displays increased *O*-GlcNAc modification in the diabetic liver (169). Normally sequestered in the cytosol through interaction with 14-3-3 proteins, this modification promotes its nuclear localization and transcriptional activation of gluconeogenesis through binding to CREB. This promotes increases in both G6Pase as well as PEPCK (**Fig. 2**). In this manner, HBP activity is proposed to increase gluconeogenesis as well as lipogenesis in the liver, promoting a viscous cycle detrimental to insulin sensitivity in fat and muscle.

I.B.5. Hexosamines and the β Cell

In addition to the liver, HBP activity also has detrimental effects on the β cells of the islets. Indeed, streptozotocin, a commonly used treatment to deplete β cell mass and recapitulate type 1 diabetes, is known to act through increasing protein

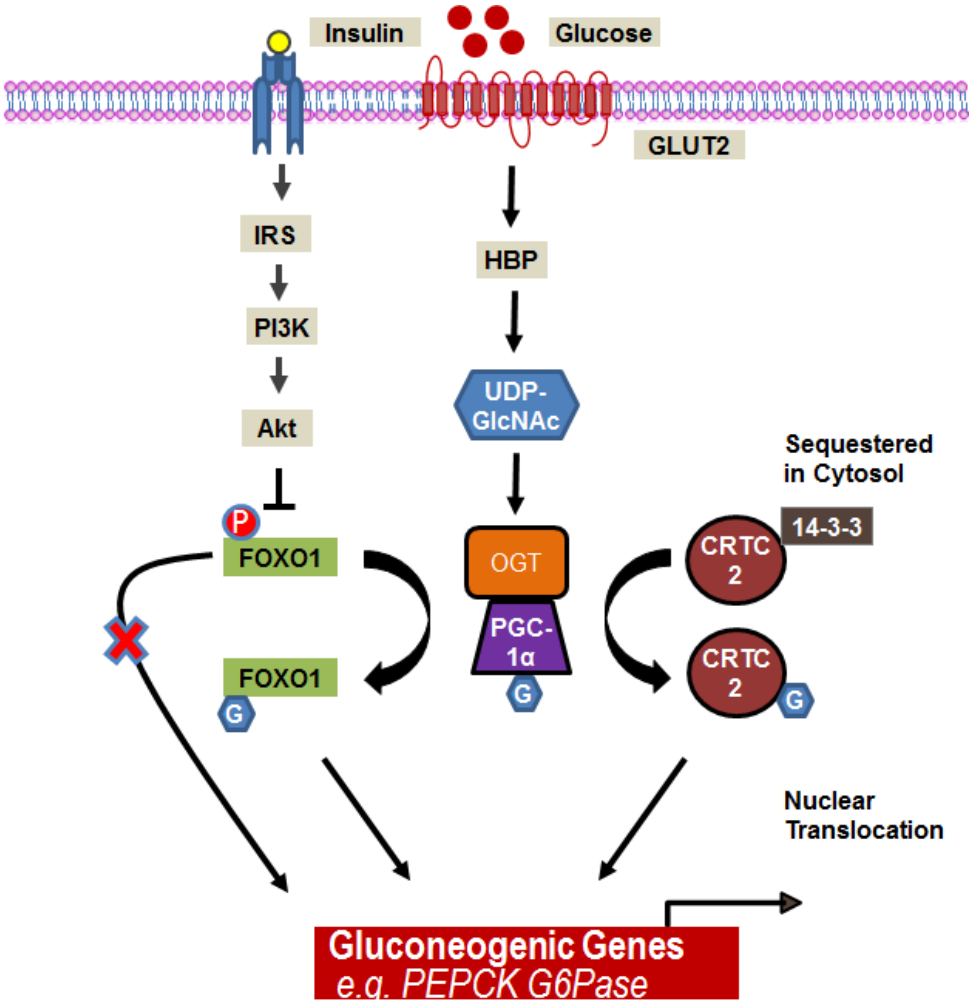

Figure 2. Schematic of the regulation of gluconeogenesis by the HBP. In the liver, insulin signaling to Akt results in the phosphorylation of FOXO1, inhibiting its entry to the nucleus and activation of a gluconeogenic program. Under conditions of excess glucose flux through the HBP, OGT mediates the glycosylation of PGC-1α resulting in the recruitment of OGT toward FOXO1. Glycosylation of FOXO1 by OGT promotes an increase in gluconeogenesis. In addition, CRTC2 is a known target of OGT. Its modification promotes the activation of both gluconeogenic genes as well as PGC-1α (see text for expanded details).

O-GlcNAc (170). In these studies INS1 cell viability was reduced by nearly 80% with 0.1 mg/ml streptozotocin or 5 mM glucosamine for 48 hours. However, other study using 0.25-1.0 mM glucosamine for 24 or 48 hours did not observe cell apoptosis but rather increased ER stress markers ccaat-enhancer binding protein (C/EBP) homology protein (Chop), spliced X-box binding protein 1 (xbp1), and activating transcription factor 6 (atf6) (171). This increase in ER stress resulted in an impairment in the functionality of the β cells as assessed by oxidative stress and the generation of reactive oxygen species (ROS), which resulted in the dedifferentiation of the β cell as indicated by a loss of GLUT2, insulin, and glucokinase expression (172, 173). Interestingly, transgenic mice that overexpress OGA have increased serum insulin and total islet insulin content, with increased angiogenesis and Akt activation (174). This supports the notion that HBP activity may impart deleterious effects on the β cell by affecting cell survival. Taken together, study has demonstrated that HBP activity can be particularly toxic to the insulin secreting β cells and may ultimately contribute to the failed compensatory response of these cells observed during the development of insulin resistance.

I.B.6. Hexosamines and Skeletal Muscle and Adipose Tissue

The mechanisms whereby elevations in HBP activity provoke insulin resistance in skeletal muscle and adipose tissue, however, are less precisely understood. In adipocytes and skeletal muscle, study has demonstrated that chronic flux through the HBP promotes the targeting of OGT to the PM through interaction with phosphoinositides, resulting in attenuation of insulin signaling

(175-177). Published data from our lab suggest increased HBP activity as a basis for palmitate, hyperglycemia, glucosamine, and hyperinsulinemia-induced losses in insulin responsiveness (7, 11, 19, 22, 178). In these studies, inhibition of the HBP with 6-diazo-5-oxo-L-norleucine (DON) protected against the development of insulin resistance. Mechanistically, this work established that excess HBP activity resulted in a loss of PIP2/F-actin structure at the PM. Concurrently, a gain in PM cholesterol content was observed. Interestingly, removal of the excess PM cholesterol with methyl-β-cyclodextrin normalized PIP2/F-actin levels and restored insulin responsiveness (7). In addition, blockade of the HBP with DON inhibited the gain in PM cholesterol and glucose transport dysfunction. One key focus of my thesis research was to determine the mechanisms by which HBP could promote these perturbations in PM cholesterol content, contributing to the development of insulin resistance.

I.B.7. Cholesterol Regulation

The maintenance of tightly regulated cholesterol levels is essential for cellular function. Approximately 65-90% of cholesterol is found at the PM, whereas the remaining 10-35% is found in intracellular membranes (179, 180). First identified by Brown and Goldstein, synthesis of cholesterol is primarily coupled to the sterol regulatory element binding protein (SREBP) (181). There exist three different isoforms of these proteins, SREBP-1a, SREBP-1c, and SREBP-2. These factors are known to reside in the endoplasmic reticulum (ER) bound in a complex with SREBP cleavage-activating protein (SCAP) and insulin inducible gene (Insig). When sterol levels are high in the cell (>5% of total ER

lipids), cholesterol binds to SCAP resulting in a conformational change that promotes its association with Insig, thereby retaining SREBP in the ER. Under conditions of low cholesterol, SCAP/SREBP is transported to the Golgi, where it is cleaved by proteases into its active form. This active form is then translocated to the nucleus where it can activate transcription of genes involved in cholesterol synthesis, such as HMG-CoA reductase (HMGR), the rate-limiting enzyme in cholesterol synthesis. This activation can also promote an increase in the levels of the low density lipoprotein (LDL) receptor, thereby promoting cholesterol uptake from the environment. In addition to regulation by cholesterol, insulin is known to promote the activation of SREBP proteins through increasing the degradation of mRNA encoding Insig (182). In this manner, the total amount of Insig protein can become rate-limiting with regard to the retention of SCAP/SREBP in the ER (183). Further, insulin stimulation can also result in the phosphorylation of SREBP, increasing the affinity of the SREBP/SCAP complex for Sec23/24 proteins of coat protein complex II (COPII) vesicles and thereby promoting transport to the Golgi for cleavage (182).

Extending beyond SREBP, HMGR activity is also known to be regulated through its direct binding to SCAP. However, dissimilar to SREBP retention, during high levels of sterols, complex formation between HMGR and Insig in the ER promotes the ubiquitination and subsequent proteasomal degradation of HMGR (184). It should be noted that these methods of cholesterol regulation are primarily mediated by SREBP-2, yet SREBP-1 plays a role in cholesterol synthesis. Transgenic mice overexpressing SREBP-1a, for instance, have a 37 fold increase in expression of HMGR in liver compared to wild-type mice (185-

187). In addition, SREBP1c, known to play a minor role in cholesterol synthesis, has increased expression in response to insulin in liver, fat and skeletal muscle (188-191).

In addition to regulating synthesis and uptake of cholesterol through the environment, cells are also able to mediate cholesterol efflux processes to moderate cholesterol levels. This process involves the ATP-binding cassette transport proteins (ABCA1 and ABCG1) (192-194). The ABCA1 transporter is known to promote unidirectional transport of free cholesterol to lipid-poor apolipoprotein (ApoA-I), forming pre-β-1 HDL. After forming this subparticle, the enzyme lecithin-cholesterol acyltransferase (LCAT) can esterify this free cholesterol generating the mature pre-β-2 or α-HDL particles (195). In contrast to ABCA1, ABCG1 is less specific and can efflux cholesterol to more mature HDL particles or even LDL particles (196). The synthesis of these transporters is reported to occur when there is an elevation in cholesterol synthesis intermediates or oxysterols, oxidized derivatives of cholesterol. This results in an activation of the liver X receptor (LXR) which can heterodimerize with the retinoic X receptor (RXR) and bind to LXR response element sequences in DNA to promote the transcription of ABCA1 other cholesterol transporters (197-200). In addition to cholesterol efflux, adipose tissue is capable of storing large amounts of non-esterified cholesterol. In fact, it is estimated that approximately 50% of cholesterol is stored in fat tissue with obesity (201). While these mechanisms tightly regulate cholesterol levels, study strongly suggests that cholesterol can become deregulated, leading to insulin resistance.

I.B.8. The HBP and Cholesterol Dysregulation

A primary component of my thesis work was the attempt to determine how hyperinsulinemia, through engaging the HBP, could promote cholesterol dysregulation despite several of the numerous regulatory processes described in the previous section. Study by the late Jeffrey Kudlow elucidated that the HBP induces *O*-GlcNAc on specificity protein 1 (Sp1), a key transcription factor involved in cholesterol synthesis (202). While normally a ubiquitous transcription factor that recognizes a GC box in the promoter of TATA-less genes (203, 204), this modification is thought to specifically target Sp1 to genes involved in metabolic processes. Much like the effect of this modification on other proteins, *O*-GlcNAc of Sp1 increases its stability and promotes its nuclear translocation (202, 205). It should be noted that phosphorylation of Sp1 on PEST sequences, (sequences rich in Pro, Glu, Ser, and Thr), targets Sp1 for degradation and that *O*-GlcNAc may occur on these same sequences, suggesting that *O*-GlcNAc may oppose the actions of phosphorylation on protein regulation (206). In terms of cholesterol synthesis, Sp1 GC binding motifs are known to be present adjacent to sterol response elements (SRE) (207, 208). The promoter of the SREBP1 is known to contain 5 Sp1 binding sites which, in conjunction with SREBP1 itself as well as LXR and NF-Y drive its maximal expression (209-212). Interestingly, a Sp1-like binding site has recently been proposed in the promoter region of HMGR (213, 214). In this context, *O*-GlcNAc on Sp1 could potentially result in increases in SREBP1 and HMGR through increasing their promoter activity. Further, increases in SREBP1 could also increase HMGR as it binds to a SRE in the promoter of HMGR. An additional recent finding is that LXR is also a target of

O-GlcNAc, with evidence suggesting the modification of this protein drives SREBP1 expression (215). Furthermore, histone H2B was also recently identified as a target of *O*-GlcNAc, driving increased expression of numerous metabolic genes including HMGR (216) In this context, my thesis work sought to determine the mechanism by which *O*-GlcNAc on Sp1 by hyperinsulinemia could modify its activity as well as provoke a cholesterolgenic response. Of interest is whether inhibition of Sp1 and/or its modification could prevent against the development of an insulin resistant phenotype.

I.B.9. Lipoprotein Metabolism and Insulin Resistance

Insulin resistance and metabolic syndrome are closely associated with dyslipidemia and alterations in circulating as well as cellular cholesterol levels. Specifically, insulin resistance often presents with high circulating levels of LDL (>100 mg/dl) and low HDL (<40 mg/dl). These findings suggest that insulin resistance is tightly coupled to alterations in lipoprotein metabolism. A focus of my thesis research aims to determine how hyperinsulinemia may promote alterations in the generation of HDL in adipocytes. While adipose tissue is primarily considered a cholesterol sink, reverse cholesterol transport (RCT) from this tissue in insulin resistant states may substantially contribute to whole body cholesterol homeostasis. In addition, parallel experiments with macrophages demonstrate very similar rates of ABCA1-mediated cholesterol efflux from these cells to ApoA1 (217). Mechanistically, ABCA1 trafficking to the membrane is facilitated by the endosomal membrane (EM) to cytosolic cycling of Rab8 in adipocytes (218). In the context of hyperinsulinemia-induced dysregulation of

cholesterol synthesis, increased endosomal cholesterol content could impair the ability of Rab8 to be extracted from the endosomal membranes by guanine nucleotide dissociation inhibitor (GDI). In fact, in patients with Niemann-Pick Disease, Type C (NPC), elevations in EM cholesterol promote the sequestering of ABCA1 (219). Additionally, Cr^{3+} supplementation in brewer's yeast has also been shown in some human trials to be necessary for the maintenance or even improve HDL levels (220, 221).

Extending beyond adipose tissue, cholesterol efflux transporters are known to play a vital role in the maintenance of cholesterol regulation in the β cells. Study has previously shown, for instance, that inactivation of the ABCA1 transporter in these cells promotes cholesterol accumulation in secretory vesicles and abrogates glucose-stimulated insulin secretion (222). Further, loss of ABCG1 has been shown to alter insulin granule morphology, cholesterol levels, and lead to impaired insulin secretion (196). In mouse models of diabetes and dyslipidemia, increases in islet cholesterol content have been observed, suggesting a potential but important role of the precise regulation of cellular cholesterol content in the maintenance of glucose sensing and insulin secretion (223). In this regard, depletion of cholesterol content in the β cells using a squalene epoxidase inhibitor, NB598, or methyl-β-cyclodextrin impaired glucose-stimulated insulin secretion (224, 225), yet treatment with lovastatin has been shown to result in impaired regulation of insulin secretion as indicated by constitutive basal secretion (226). Together, the role of Cr^{3+} action on improving lipoprotein metabolism is currently poorly understood. The following subsection will cover a brief history of Cr^{3+} and its impact on glucose and lipid metabolism in

health and disease. It will also outline several proposed mechanisms for Cr^{3+} action in adipose and skeletal muscle tissue.

I.C. Chromium Supplementation in Health and Disease

The case for Cr^{3+} as beneficial in the maintenance of healthy glucose metabolism was first established in 1957 when Schwarz and Mertz discovered a concentrate from brewer's yeast that improved glucose tolerance in Sprague-Dawley rats (227). While initially deemed a glucose tolerance factor, its isolation and identification as Cr^{3+} was determined just two years later (20, 228). Shortly after its identification, it was determined that the site of action of this nutrient was the plasma membrane. Initially, autopsy results demonstrated that Cr^{3+} levels severely declined in tissues with age, particularly in the heart where it was reduced by greater than 95% by age 20 (229). This finding ultimately led to the hypothesis that chromium deficiency could promote the development of atherosclerosis. Indeed, decades later Cr^{3+} deficiency is known to promote increases in serum cholesterol, triglycerides, and decreased levels of HDL (221, 230, 231). In humans, even after its discovery, trivalent Cr^{3+} was still universally considered a toxic carcinogen, although less so than hexavalent Cr^{6+}. This notion disappeared when it was recognized that the biologically essential trivalent form could not be oxidized in living organisms to the carcinogenic hexavalent form (228). Yet the essentiality of Cr^{3+} was still heavily debated due to the fact that Alan Walsh's invention of atomic absorption, used to detect intestinal Cr^{3+} absorption, placed the rate at close to zero. As analytical methods improved,

however, the essentiality of Cr^{3+} in animals for optimal glucose and lipid metabolism became accepted.

In 1977, Cr^{3+} was declared as an essential nutrient for humans when a woman receiving total parenteral nutrition (TPN) devoid of Cr^{3+} presented to a physician with severe weight loss, peripheral neuropathy, and hyperglycemia (232). An intravenous glucose tolerance test on this patient confirmed that she was glucose intolerant. Administration of 45 units of insulin was required to maintain plasma glucose values at normal ranges, although this amount of insulin did not mitigate the glucose intolerance. Based on emerging evidence that chromium deficiency could provoke glucose intolerance, levels of chromium in the blood and hair were assessed and it was determined that there was approximately a 90% reduction in the levels compared to normal values. Subsequent addition of Cr^{3+} to the nutrition regimen over the course of several weeks resulted in a significant potentiation of insulin's action on glucose tolerance and reduced the amount of insulin required in this patient to 15 units.

Since then, numerous studies have shown a beneficial effect of Cr^{3+} on at least one parameter of glycemia in humans (233-238). Furthermore, a recent systematic review of the randomized control trials with chromium found that chromium improved glycosylated hemoglobin as well as fasting glucose (23). The authors conclude based on the current research that chromium may have modest effects on glucose sensitivity. They do, however, point out that of the trials that found no significant effect of chromium, nearly half of them were poorly controlled and/or statistically underpowered. Since the benefit of chromium treatment could also be masked in patients taking medications, this could

suggest a more than modest effect of chromium on patients. Of note, a majority of these findings were conducted in patients with T2D. Study by Richard Anderson, who observed large benefits of chromium in patient populations, witnessed an absence of a beneficial effect in individuals with normal glucose tolerance (239). In fact, an increase in 90 minute post-load glucose levels was seen in these patients. However, another study of the short term effects of chromium in overweight children found a significant improvement in HOMA (240). Complicating interpretation further, study by William Cefalu has suggested that patient phenotype is important in the clinical response to chromium supplementation (234, 241). Clinically, a parameter has not yet been determined to assess patients who have a low chromium status (to be discussed below). However, a future direction suggested of clinical trials is to delineate precisely what patient populations would benefit from supplementation, providing individualized care (242). Furthering this endeavor will undoubtedly require mechanistic details into the actions of chromium to better understand what metabolic abnormalities that present in patients could be best amendable to chromium therapy.

I.C.1. Chromium Balance and Insulin Resistance

An important consideration with regard to human studies is the precise amount of chromium needed to evoke a physiological response. As such, a wealth of studies have been conducted with regard to the bioavailability of chromium as well as the daily requirements. As recommended by the Food and Nutrition Board of the Institute of Medicine of the National Academy of Sciences,

the adequate intake of chromium is set to 30 µg/d (243). Given the known absorption of chromium at approximately 0.5%, this presents 0.15 µg to the bloodstream per day. Retrospectively, this indicates that in cases like the woman presented above receiving total parenteral nutrition devoid of chromium, the amount of chromium delivered was perhaps “adequate”. As John Vincent describes, given the concentrations in the TNP that were provided, the solutions ranged from 2-6 µg/d. While initially considered devoid, the intravenous diet ensured that all of the chromium was delivered into the bloodstream, meaning that patients were actually receiving more than the 0.15 µg/d that is considered adequate (232, 244-246). In addition, the levels of chromium added back to the TPN were actually 125-250 µg/d, a pharmacological dose. In humans, studies have provided at maximum a dose of 1 mg/d. Based on safety recommendations stating that up to 10 mg/day of chromium would be expected to have no health risks, the need for clinical trials aimed at using higher doses of chromium for long periods of time while carefully monitoring for adverse risks would greatly benefit clinical trials.

In this regard, there are several different forms of chromium commercially available. These include chromium yeast (CrY), chromium chloride ($CrCl_3$), and chromium picolinate (CrPic), the most stable form (247). Recent other pharmacological chromium complexes known to exert beneficial effects on glucose and lipid metabolism have also been generated, including chromium nicotinate/niacin (CrN), chromium (D-phenylalinine$_3$), and chromium citrate (CrCIT) (248-250). While CrPic is popularized because of its known greater absorption (251), coupled to its increased excretion, its bioavailability is not

thought to be greater than other forms available (251, 252). Nevertheless, it retains its popularity, as CrPic containing supplements are currently ranked second in sales among mineral supplements after calcium (253).

In the context of insulin resistance, it is known that alterations occur in the urinary excretion of chromium. In fact, in non-insulin resistant type 1 diabetic children, study has shown decreased plasma, erythrocyte and urine chromium compared to non-diabetic control individuals (254). In another study, increased insulin resistance in non-diabetic Saudi adults was highly correlated with increased excretion of chromium in the urine, suggestive of chromium deficiency (255). In diabetic rats, which also demonstrated increased urinary chromium loss as a result of diabetes, there is also a compensatory increased absorption which is thought to balance chromium status, again suggesting that beneficial effects of chromium may not come from alleviating chromium nutritional deficiency but rather supplementation at pharmacological doses (256). Nevertheless, some studies still suggest that the typical western diets do not provide enough chromium for the adequate daily intake requirement (247, 257-259), so the possibility that a deficiency exists that could be amendable to replacement cannot be discredited.

I.C.2. Cellular and Mechanistic Details of Chromium Action

Several mechanisms have been proposed to explain the details of chromium's effect on potentiating insulin action. For example, chromium has been shown in studies increase insulin binding to its receptor, the number of receptors, and also receptor phosphorylation and activation (260-262). Studies

have demonstrated that chromium is transported in the circulation bound to siderophilin (transferrin) or albumin, although >90% is proposed to be bound to transferrin (263-266). Although transferrin exhibits strong selectivity for iron (III) ions based on its binding sites, in humans the protein has been shown to normally be 30% saturated with iron (267). UV spectra analysis has subsequently demonstrated that chromium can bind to two metal sites in transferrin (268-271). The binding of chromium to transferrin has been shown biochemically to be quite strong, with an effective binding constant of 2.92×10^{15} M^{-2} (267). Upon endocytosis of transferrin by the cell, it must then be transferred from the endosome to the cytosol. Unlike iron, which is reduced and transported to the cytosol by divalent metal transporters, chromium is not readily reduced and the mechanistic details of its transport are currently a topic of future investigation.

With regard to transferrin's ability to transport both iron and chromium, a recent Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study in humans has associated increased levels of total iron, serum ferritin (the primary means of iron storage), and non-transferrin-bound iron with adipocyte insulin resistance, measured as the product of fasting insulin and nonesterified FAs (272). In another human study, it was found that circulating serum soluble transferrin receptor levels were inversely correlated with insulin sensitivity in normal glucose tolerant, non-obese subjects (273). Interestingly, however, in humans with T2D there is a reported loss in the serum levels of chromium compared to nondiabetic control subjects (274). Together, these studies suggest that, even if deficiencies are not present, alterations in the balance of circulating chromium and iron levels could contribute to the pathology of insulin resistance.

Upon entering the cell, chromium is known to bind the low-molecular-weight chromium binding substance (LMWCr, chromodulin). It has been suggested that this response is mediated by the action of insulin on the insulin receptor (275, 276). Upon binding of chromium to chromodulin, this complex then binds to the receptor and has been suggested to maintain the receptor in its active conformation, thereby amplifying the signaling cascade (246). Further study needs to be conducted to elucidate the exact biochemical mechanism of this response. In addition, little is known about the regulation of the formation of this chromodulin complex, a necessary topic of future research.

In addition to reports suggesting that chromium may improve the ability of insulin to stimulate glucose transport into adipose and skeletal muscle tissue through modulation of insulin signaling, work in our lab and others suggests chromium may positively influence PM parameters including membrane fluidity (21, 277). Furthermore, moderate increases in PM fluidity have previously been shown to increase glucose transport (278-280). Consistent with this hypothesis, reductions in membrane fluidity have been shown to impair glucose transport (279). In our adipocyte studies chromium was found to diminish excess PM cholesterol content, concomitant with an increase in the redistribution of GLUT4 to the PM. This event was lost in the presence of exogenously added cholesterol, strengthening the hypothesis that alterations in PM cholesterol contribute to chromium's action. Interestingly, insulin-stimulated glucose transport was not enhanced by chromium treatment. However, in cells treated with chromium in the presence of insulin, there was a significant increase in glucose transport. None of these observed effects were associated with amplification of insulin signaling.

Whereas these studies were performed in cells cultured in high glucose conditions (25 mM glucose), chromium did not have an effect on the redistribution of GLUT4 to the PM in cells cultured in non-diabetic (5 mM glucose) conditions (22). Recent studies in our lab also demonstrate that chromium does not improve glucose uptake in L6 myotubes grown under non-diabetic conditions. However, when cells were treated with pathophysiologic hyperinsulinemia (5 nM, 12 h) in the presence of CrPic (100 nM, 16 h), chromium restored insulin responsiveness and corrected hyperinsulinemia-induced cholesterol accrual. Taken together, this data strongly suggests a beneficial effect of chromium on membrane parameters through altering PM cholesterol content.

I.C.3. AMPK and Chromium Action

Mechanistically, recent study suggests that a target of chromium action entails activation of 5' AMP activated protein kinase (AMPK) (19, 281). Furthermore, knockdown of AMPK was demonstrated by our lab to negate the beneficial effects of chromium on GLUT4 translocation and glucose uptake in hyperinsulinemic cells (unpublished data). These studies demonstrated that an 80% knockdown of AMPK in transfected cells resulted in a loss in chromium's ability to phosphorylate and activate of AMPK as observed in scramble transfected cells. Additionally, insulin signaling was intact in the knockdown cells and chromium's mode of action did not involve amplification or correction of insulin signaling defects. Rather, they strongly suggest that chromium, through engaging AMPK, may inhibit cholesterol accrual. In addition, elevations in

membrane cholesterol were also observed in C57Bl/6J mice fed a HF diet, whereas chromium supplementation in these animals restored cholesterol content concomitant with a recovery in glucose and insulin tolerance. These studies are of interest in the context that AMPK is known to result in the phosphorylation and inactivation of HMGR. This observation could potentially explain the mitigation in cholesterol accumulation at the PM observed in cells pretreated with chromium.

Of note, AMPK has been referred as a fuel gauge sensing low energy status (282-287). This occurs when the cell has depleted high energy molecules such as 5’ adenosine triphosphate (ATP), resulting in an increase in the AMP/ATP ratio. In the absence of AMP, the complex exists in an inactive conformation and the α and γ subunits do not directly interact. In this form, the active site cleft is blocked, preventing upstream kinases from phosphorylating an important activating threonine 172 residue. When AMP accumulates in the cell, it can directly bind between the α and γ subunits at a cystathionine-β-synthase (CBS) domain, exposing the threonine site for activation (288). In this manner, AMP binding promotes a conformational change in the α catalytic subunit, rendering it susceptible to upstream kinase targeting on threonine 172, thereby triggering its activation (289-291). Two kinases have been proposed to activate AMPK in this way, including liver kinase B1 (LKB1) (292-294) and calmodulin-dependent protein kinase β (CaMKKβ) (295-297). Indeed, phosphorylation at this residue has been shown to be essential for kinase activity and serves as a good marker to assess the activity of the kinase (291, 298, 299). In addition to inhibiting HMGR, numerous studies have shown that AMPK activation regulates enzymes

essential for FA metabolism by phosphorylation of acetyl-CoA carboxylase (ACC) on serine 79, thereby attenuating the production of malonyl-CoA, a key intermediate in FA synthesis (300-308). In this manner, chromium treatment, through activation of AMPK, might work in the cell to promote energy producing pathways while simultaneously inhibiting those anabolic processes such as cholesterol and FA synthesis.

While AMPK regulation was not a major focus of my doctoral research, the role of AMPK in energy conservation and inhibition of HMGR activity is of interest. In stark juxtaposition to the HBP, a sensor of excess energy bioavailability within the cell, AMPK activity signals energy conservation pathways. As anticipated, research has recently suggested a crosstalk between these two pathways as AMPK is a known target of OGT and AMPK has also been shown to phosphorylate and inhibit GFAT (309-311). This finding is of significance as it suggests pleiotropic actions of chromium on preventing PM cholesterol accrual that impairs insulin action. As such, a hypothesis of my thesis research is that chromium may inhibit transcriptional responses promoting cholesterol synthesis, thereby restoring insulin responsiveness. The effects of chromium on these parameters were tested mechanistically *in vitro* in the context of hyperinsulinemia-induced activation of the HBP. In addition, as C57Bl/6J mice suggest increased HBP as a mechanism fueling insulin resistance, study sought to validate *in vitro* findings and delineate the mechanistic aspects of chromium treatment *in vivo*. The following subsection gives a brief report of the clinical relevance of this work in the context of current diabetes treatment options.

I.D. Clinical Perspectives

As aforementioned, the prevalence of T2D and its deleterious effects on the quality of life for patient populations represents a global health concern. Currently, 6.4% of the world population has T2D, and it is estimated that in the next 20 years the prevalence will grow by over 20% (312). While a major genetic basis exists for the development, the recent surge in prevalence underscores the influence of lifestyle, environmental, and technological factors in contributing to the diagnosis and prevalence of disease. While diet and exercise are proposed to strongly prevent the development of disease, patient compliance is a major fundamental challenge. In this regard, the use of pharmacological chromium for certain patient populations may help alleviate the burden of T2D. While clinical studies suggest a beneficial effect primarily in T2D, the modest effect of chromium could be masked by the effects of other antihyperglycemic drugs. For instance, several agents that normalize blood glucose concentrations, improve insulin action, or improve metabolic syndrome including metformin (313-315), rosiglitazone (316), troglitazone (317), exendin-4 (318), glucagon-like peptide 1 (GLP1) (319), miglitol (320), and atorvastatin (321) are reported to activate AMPK. In addition to these, numerous nutritional supplements are also shown to activate AMPK, including berberine (322-324), triterpenoids (325), and resveratrol (326-329).

While many studies have examined the potential of therapeutic targets on activation of AMPK, few have focused attention on whether any inhibit the HBP activity. Of particular interest, however, was a study which found that treatment of adipocytes with troglitizone or rosiglitazone inhibited HBP activity induced by

glucosamine treatment (330). Strikingly, treatments with these thiazolidinediones (TZDs) did not inhibit global *O*-GlcNAc, but specifically inhibited increased modification of Sp1, thereby reducing the expression of the adipokine, resistin (331). Taken together, these findings suggest that repression of HBP-induced modification of the activity of Sp1 might represent a therapeutic target that could potentially alleviate insulin resistance. These findings also underscore the importance of gaining further understanding of the mechanisms by which increased activation of the HBP and subsequent Sp1 modification may provoke insulin resistance in the context of our findings that PM cholesterol accrual may represent a key derangement contributing to insulin resistance.

I.E. Thesis Hypothesis and Specific Aims

Based on the fundamental research findings presented above and the current gap in the understanding of transcriptional mechanisms leading to cholesterol accrual and actions of chromium, I formulated the following hypotheses for my thesis research. My central hypothesis is that hyperinsulinemia-induced elevations in the HBP activate a cholesterolgenic response by modulation of critical transcription factors involved in the regulation of cholesterol synthesis. Furthermore, I predict that the beneficial effects of chromium on glucose and cholesterol homeostasis will be mediated through an inhibitory effect on HBP-induced cholesterol accrual. Using cellular and animal models of insulin resistance, I tested my central hypothesis by pursuing the following two specific aims: 1) Dissect the transcriptional mechanisms of aberrant cholesterol synthesis promoting insulin resistance and 2) Delineate the protective

actions of chromium supplementation on the HBP and cholesterol efflux processes.

Chapter II. Results

II.A. Increased HBP Activity Provokes Cholesterol Synthesis, Cytoskeletal Dysfunction, and Insulin Resistance via Transcriptional Activation of Sp1

II.A.1. Summary

Increased caloric intake and/or obesity are currently the greatest predisposing risk factors for the development of T2D. Recent study implicates increased nutrient flux through the HBP as an underlying basis for the development and exacerbation of insulin resistance and β cell failure, hallmark events in the pathology of T2D. Nevertheless, the precise mechanistic insight into how excessive HBP flux contributes to the desensitization of the glucose transport system is currently unknown. Previous study has demonstrated that hyperinsulinemia-induced increases in HBP activity resulted in PM cholesterol accumulation and insulin resistance in 3T3-L1 adipocytes. Here, we tested the effects of increased HBP activity on provoking a transcriptional cholesterolgenic response involving the modification of Sp1, potentially promoting increases in its binding affinity and activity toward promoter regions of SREBP1 and HMGR. In 3T3-L1 adipocytes, varying low, pathophysiological doses of insulin (250 pM - 5 nM) for 12 h promoted increased PM cholesterol as well as HMGR mRNA/protein. SREBP1 was increased under these conditions, both in its inactive and active, nuclear forms. *O*-GlcNAc modification of Sp1 was elevated in cells treated with hyperinsulinemia, whereas HBP inhibition with DON protected

against this effect. This modification of Sp1 was associated with a greater affinity of this transcription factor toward the promoter of HMGR and SREBP1. Luciferase assays confirmed that this increase in binding resulted in activation of the promoter. Treatment with DON prevented against these alterations as well as the increased mRNA/protein content of HMGR. Consistent with the role of Sp1 in mediating these effects, the Sp1 DNA-binding inhibitor, mithramycin, completely rescued increased HMGR protein, PM cholesterol, F-actin loss, and glucose transport dysfunction. These data suggest a possible mechanism whereby the modern lifestyle, abundant in excess nutrient intake, may impinge on glucose transport and contribute to the development of T2D.

II.A.2. Results

Pathophysiological Hyperinsulinemia Provokes Cholesterol Synthesis: Studies have demonstrated that hyperinsulinemia induces cellular insulin resistance via increased PM cholesterol content, in turn reducing cortical F-actin necessary for distal components of GLUT4 translocation (7, 11) As pathophysiological hyperinsulinemia has been defined as levels of insulin between 186 pM and 4.2 nM (135), it was probed whether low doses of insulin would increase PM cholesterol content in 3T3-L1 adipocytes. Overnight exposure of cells to 250 pM, 500 pM, and 5 nM insulin resulted in a 20%, 45% and 43% increase in PM cholesterol, respectively, compared to control cells (**Fig. 3A**). While all 3 doses significantly increased PM cholesterol, the 500 pM and 5 nM doses resulted in increases that were significantly different than that observed

with the 250 pM dose of insulin. The effects of these doses of chronic insulin on the status of HMGR were next assessed. Consistent with hyperinsulinemia modulating the transcriptional activity of the gene, 250 pM, 500 pM and 5 nM insulin all increased the mRNA expression of *Hmgcr*, encoding HMGR (**Fig. 3B**). This was consistent with an observed gain in HMGR protein levels in cells exposed to 500 pM and 5 nM, but not 250 pM insulin (**Fig. 3C**). As HMGR is known to be regulated by SREBP1 and SREBP2, total levels of these proteins were examined. Treatment with 500 pM and 5 nM insulin resulted in an 80% increase in total SREBP1 content (**Fig. 3D**). SREBP2 protein content was unaffected by any of these treatments. In all of these experiments, the 500 pM dose of insulin had the same effect as the 5 nM dose. As such, all subsequent studies utilized this dose to examine the effects of the HBP on cholesterol synthesis.

Hyperinsulinemia Engages the HBP to Promote *O*-linked Glycosylation of Sp1: It has been previously documented that flux through the HBP increases *O*-GlcNAc of Sp1 (332). Study next sought to determine if hyperinsulinemia resulted in an increase in modification of this protein. Immunoprecipitates of Sp1 from insulin-treated adipocytes displayed a marked increase in *O*-linked glycosylation compared to control cells (**Fig. 4A**). In contrast, concurrent treatment with the HBP inhibitor DON completely prevented against this elevation in Sp1 *O*-GlcNAc. In addition, treatment with DON had no effect on this parameter in control cells. To test the sensitivity of the RL2 antibody in detecting changes in glycosylation status of Sp1, a Click-iT *O*-GlcNAc enzymatic labeling

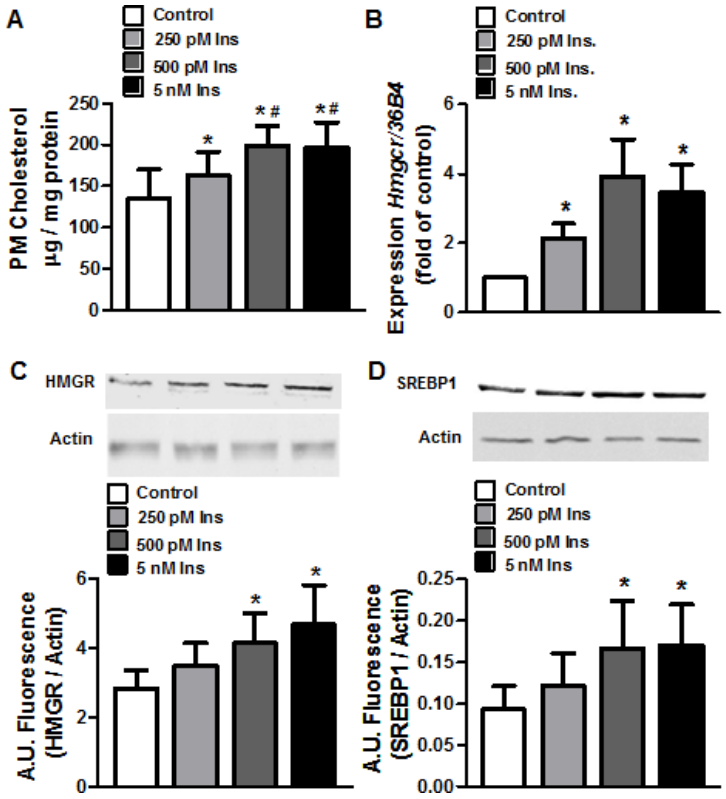

Figure 3. Pathophysiological hyperinsulinemia provokes a cholesterolgenic response. 3T3-L1 adipocytes were treated with or without 250 pM, 500 pM, or 5 nM insulin for 12 h. PM cholesterol content (A) was assessed and normalized to total PM protein content. Mean values ± SEM are shown from 4 independent experiments. *, P<0.05 versus control. #, P<0.05 versus 250 pM insulin. The mRNA content of *Hmgcr* (B) was also determined and normalized to *36B4*. Means values ± SEM are shown from 8-9 independent experiments. *, P<0.05 versus control. Protein levels of HMGR (C) were determined by immunoblotting, normalized to actin. Mean values ± SEM are shown from 6 independent experiments. *, P<0.05 versus control. Total protein levels of SREBP1 (D) were also determined by immunoblotting, normalized to actin. Mean values ± SEM are shown from 3 independent experiments. *, P<0.05 versus control.

system was used for a more sensitive detection of the hyperinsulinemia-induced and DON-inhibited increases in *O*-GlcNAc modification. While the trends observed were similar, the Click-iT kit revealed an approximate 3 fold increase in modification of Sp1 with hyperinsulinemic conditions (**Fig. 4B**). While *O*-linked glycosylation has been observed to promote an increase in its nuclear localization, nuclear Sp1 was not elevated with hyperinsulinemia, consistent with other reports that this factor is mainly localized to the nucleus (149, 333). However, hyperinsulinemia did result in a 54% increase in SREBP1 content in nuclear fractions (**Fig. 5**). Consistent with the HBP provoking this gain in nuclear SREBP1, DON treatment strongly trended (P=0.07) to prevent this increase. HBP inhibition had no effect on localization of SREBP1 under control conditions.

Hyperinsulinemia-induced *O*-GlcNAc of Sp1 Increases its Binding Affinity: To determine if elevated glycosylation of Sp1 induced by hyperinsulinemia resulted in the transactivation of cholesterolgenic genes such as *Srebf1*, encoding SREBP1 and *Hmgcr*, encoding HMGR, chromatin immunoprecipitation (ChIP) was performed. As previous studies have identified that many SRE responsive genes contain binding sites for Sp1, including *Srebf1*, the binding affinity of Sp1 toward this promoter was first examined (207, 208, 334, 335). Results from these assays revealed that hyperinsulinemia treatment induced an approximate 2 fold increase in the binding affinity of Sp1 toward the promoter region of SREBP1 (**Fig. 6**). In control cells, very little binding was detected and DON did not affect this affinity. However, the hyperinsulinemia-induced increase in Sp1 binding toward the promoter was abrogated by blocking

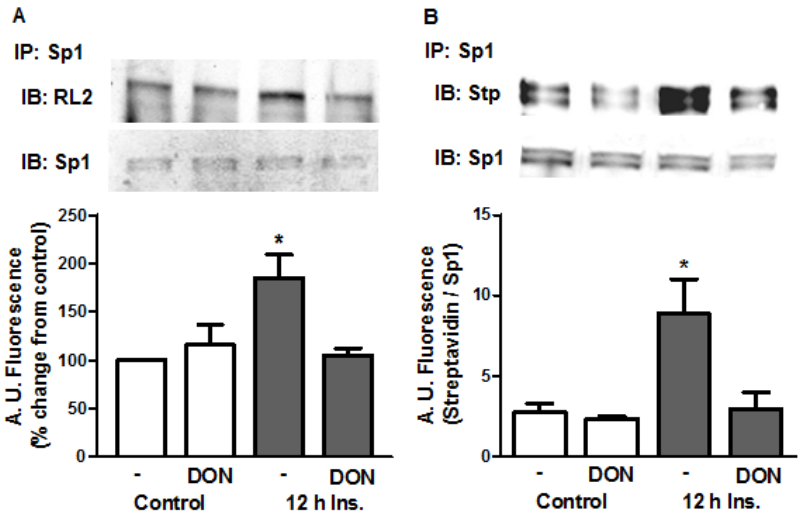

Figure 4. Hyperinsulinemia provokes *O*-linked glycosylation of Sp1. 3T3-L1 adipocytes were pretreated with or without 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, lysates were prepared and immunoprecipitated with an Sp1 antibody. Eluted samples were then immunobloted with an RL2 antibody (A) to detect *O*-linked glycosylation of Sp1. The glycosylation of Sp1 was normalized to an input sample of Sp1 from lysed samples. Means values ± SEM are shown from 4 independent experiments. *, $P<0.05$ versus control. Immunoprecipitates prepared as described above were subjected to enzymatic labeling with a Click-iT kit and immunobloted with a streptavidin (Stp) antibody (B). Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

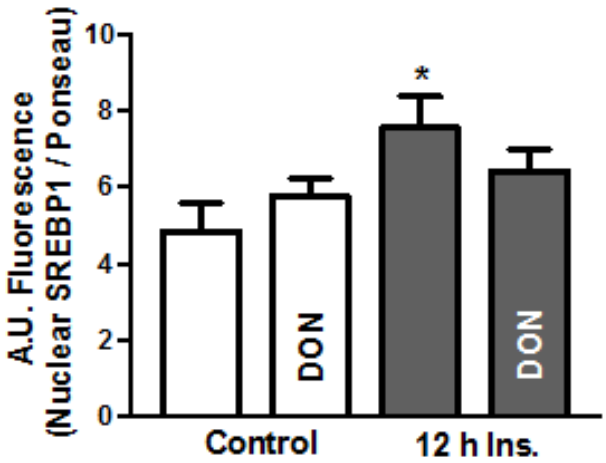

Figure 5. Hyperinsulinemia provokes nuclear localization of SREBP1. 3T3-L1 adipocytes were pretreated with or without 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, nuclear fractions were prepared from lysates. Nuclear extracts were then immunoblotted with an antibody for SREBP1. The total nuclear content of SREBP1 was normalized to Ponceau staining. Means values ± SEM are shown from 4 independent experiments. *, $P<0.05$ versus control.

the HBP with DON. Interestingly, an Sp1-like binding site in the promoter region of HMGR has also been observed (213). ChIP analyses were thus conducted to assess the binding affinity of Sp1 toward this promoter under these conditions. Similarly, basal binding of Sp1 to HMGR was minimal, yet hyperinsulinemia demonstrated a 4 fold increase in the binding of Sp1 toward this promoter (**Fig. 7**). This effect was lost upon concurrent treatment with DON.

Hyperinsulinemia Promotes the Transcriptional Activation of HMGR: Since the transcriptional regulation of SREBP1 by Sp1 has been studied extensively (210, 211, 336), whereas regulation of HMGR by Sp1 is incompletely understood, study sought to determine the role of the Sp1 binding site in the promoter region of HMGR. Specifically, it was sought to determine how hyperinsulinemia, which provoked an increase in binding of Sp1 toward the promoter, affects the activity of this promoter. Plasmids which contained the coding sequence of HMGR coupled to luciferase (**Fig. 8A**) were electroporated into 3T3-L1 adipocytes and luciferase assays were performed after treatments. This promoter region was determined to contain the 3 consensus binding sites for Sp1 that were determined by *in silico* analysis using MatInspector (**Fig. 8B**). In cells chronically exposed to 500 pM insulin, the activity of the promoter region of HMGR was found to be elevated by approximately 50% compared to control cells (**Fig. 8C**). Consistent with the ChIP data showing a reduction in Sp1 binding to the promoter when cells were treated with hyperinsulinemia in the presence of DON, the increased luciferase activity was corrected with DON treatment. Together, these results suggest that hyperinsulinemia-induced *O*-linked

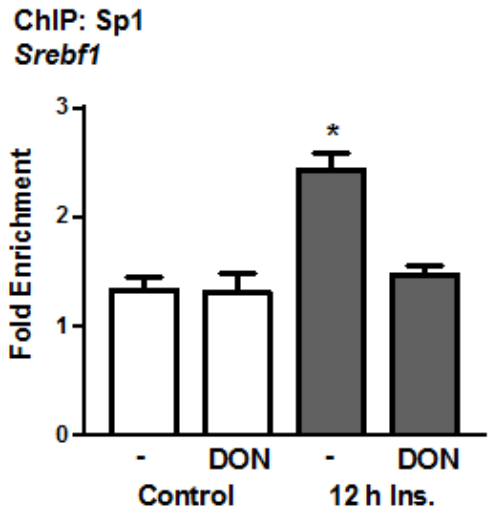

Figure 6. Hyperinsulinemia increases Sp1 binding to the promoter of SREBP1. 3T3-L1 adipocytes were pretreated with or without 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, DNA was sonicated and ChIP was performed using an Sp1 antibody. Purified DNA and primers specific to the Sp1 binding site in the promoter region of SREBP1 were utilized for qPCR analyses. Ct values from qPCR were normalized to the background IgG antibody using the fold enrichment method. Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

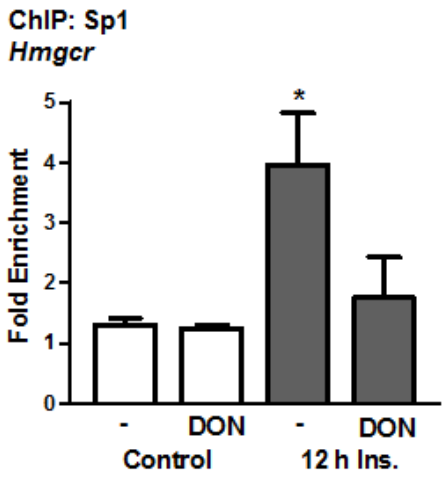

Figure 7. Sp1 binds to the promoter region of HMGR and its insulin-induced increased affinity is prevented by DON. 3T3-L1 adipocytes were pretreated with or without 20 μM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, DNA was sonicated and chromatin immunoprecipitation was performed using an Sp1 antibody. Purified DNA and primers specific to the Sp1 binding site in the promoter region of HMGR were utilized for qPCR analyses. Ct values from qPCR were normalized to the background IgG antibody using the fold enrichment method. Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

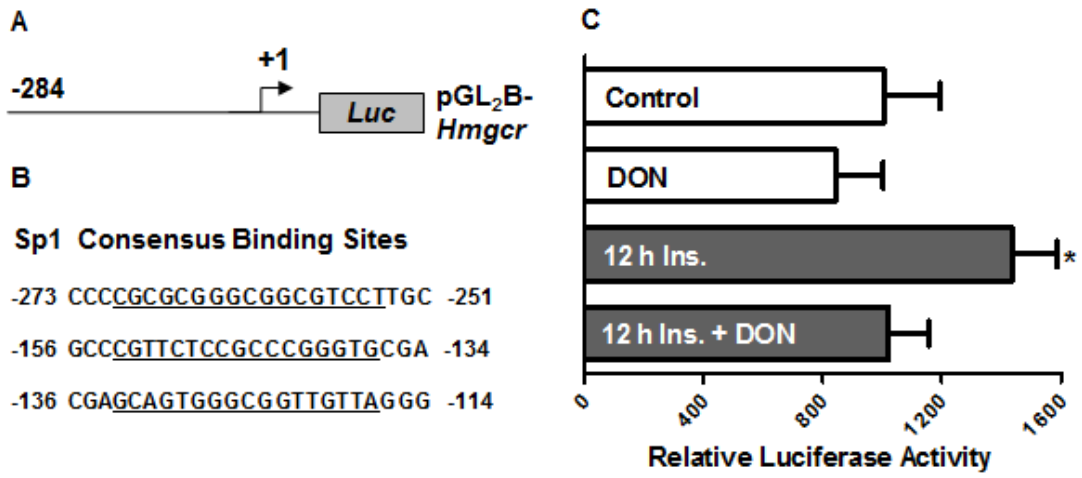

Figure 8. Hyperinsulinemia Promotes and DON Protects Against Transcriptional Activation of the HMGR Promoter. 3T3-L1 adipocytes were electroporated with the proximal promoter sequence (-284 to +36) of the HMGR promoter cloned into pGL2B luciferase reporter plasmids (A). This promoter sequence contained 3 consensus sequences for Sp1 binding (B). After a 16-18 h recovery period, cells were pretreated with or without 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, luciferase activity was measured and normalized relative to Renilla (C). Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

glycosylation of Sp1 results in increased binding and activation of these genes, in turn, promoting an increase in cholesterol synthesis and PM cholesterol content.

Inhibition of the HBP Ablates the Cholesterolgenic Response: It was next determined whether DON could protect against the increases in mRNA/protein content of HMGR observed with hyperinsulinemia. HMGR mRNA obtained from cells treated with 500 pM insulin displayed a characteristic 4 fold increase in mRNA compared to control cells (**Fig. 9A**). Treatment with DON completely abrogated the insulin-induced increase in HMGR mRNA. Interestingly, in control cells there was a slight, but significant lessening of the mRNA levels of HMGR. The reduction observed was approximately 40% compared to control cells. In addition to measuring mRNA, the effect of DON on the protein content of HMGR was also assessed. While hyperinsulinemia promoted a 55% increase in protein levels of HMGR (**Fig. 9B**), DON protected against this alteration. In contrast to that observed with the mRNA data, the decrease in mRNA levels of HMGR witnessed in control cells did not amount to a detectable loss in total protein content.

Sp1 Inhibition Protects Against Hyperinsulinemia-induced Glucose Transport Dysfunction: Taken together, this data suggested that hyperinsulinemia, through engaging the HBP, could promote an increase in cholesterol synthesis through alterations in the glycosylation status of Sp1. Study thus next sought to decipher the precise role of Sp1 in mediating these transcriptional alterations. As such, a specific inhibitor of Sp1 binding to GC box

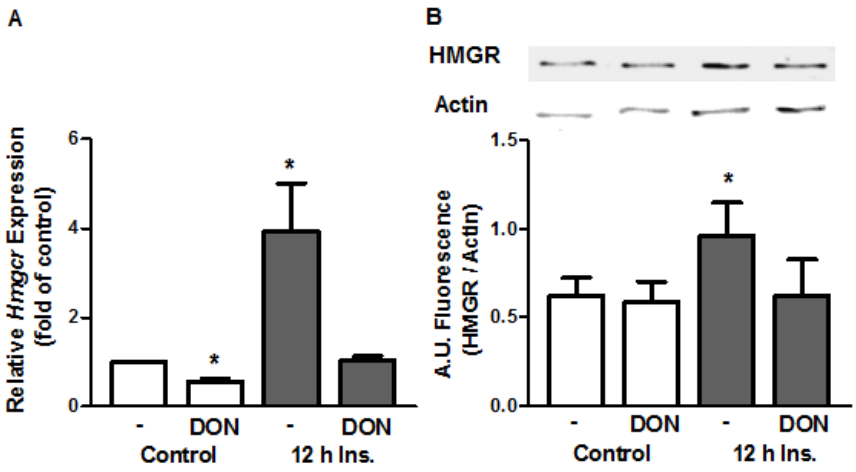

Figure 9. Inhibition of the HBP pathway protects against the cholesterolgenic response. 3T3-L1 adipocytes were pretreated with or without 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, the mRNA content of *Hmgcr* (A) was determined and normalized to *36B4*. Means values ± SEM are shown from 3-8 independent experiments. *, P<0.05 versus control. Protein levels of HMGR (B) were also determined by immunoblotting, normalized to actin. Means values ± SEM are shown from 5 independent experiments. *, P<0.05 versus control.

regions in the DNA, mithramycin, was employed (337). Protein levels of HMGR were elevated in cells treated with hyperinsulinemia, but corrected in cells treated in the presence of mithramycin (**Fig. 10A**). In addition, PM cholesterol content was increased by approximately 35% under hyperinsulinemic conditions. This increase, however, was protected against when cells were cotreated with mithramycin (**Fig. 10B**). To next assess if mithramycin could correct against a loss in F-actin previously observed in cells cultured in hyperinsulinemic conditions, confocal microscopy was employed. In cells treated with hyperinsulinemia, there was a marked qualitative loss in F-actin staining as assessed with a phalloidin compared to control cells (**Fig. 11A**). This apparent loss in staining was corrected in cells treated with hyperinsulinemia in the presence of mithramycin. Quantification of the actin staining using MetaMorph software, normalized to Syto 60, a nuclear stain, revealed a 55% loss in F-actin that was corrected with mithramycin treatment (**Fig. 11B**). Finally, it was determined whether mithramycin treatment, through correcting these membrane/cytoskeletal derangements, could protect against an impairment in insulin responsiveness induced by hyperinsulinemia. Glucose uptakes revealed that hyperinsulinemia impaired the acute ability of insulin to stimulate glucose uptake by approximately 50% compared to control cells (**Fig. 12**). In contrast, insulin-stimulated glucose uptake was not impaired by mithramycin treatment of control cells. Rather, mithramycin restored insulin responsiveness in cells treated with hyperinsulinemia.

In summary, data from these *in vitro* studies presented in Chapter IIA offer several novel observations. First, accompanying the hyperinsulinemia-induced

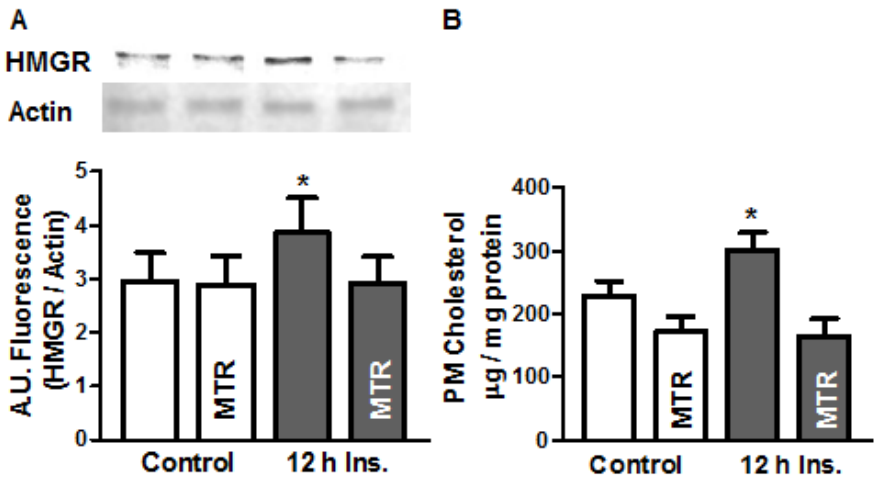

Figure 10. Inhibition of Sp1 binding activity protects against cholesterol accrual. 3T3-L1 adipocytes were pretreated with or without 100 nM mithramycin (MTR) for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, the protein levels of HMGR (A) were determined by immunoblotting, normalized to actin. Means values ± SEM are shown from 4 independent experiments. *, P<0.05 versus control. PM cholesterol content (B) was also assessed and normalized to total PM protein content. Mean values ± SEM are shown from 5 independent experiments. *, P<0.05 versus control.

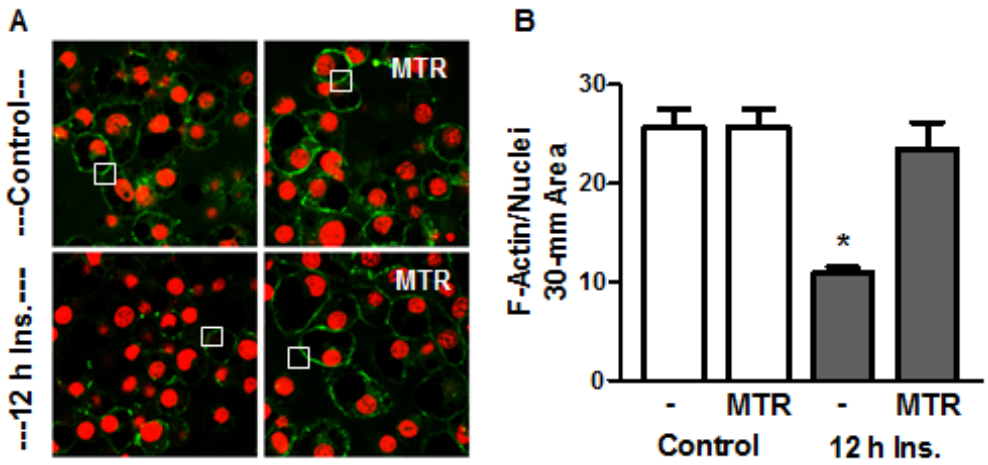

Figure 11. Inhibition of Sp1 binding activity protects against F-actin loss. 3T3-L1 adipocytes were pretreated with or without 100 nM mithramycin (MTR) for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, cells were fixed and subjected to immunofluorescence microscopy with phalloidin for F-actin imaging and a Syto 60 nucleic stain (A). All microscope settings were identical between groups. Quantification of signal intensity from X60 magnification fields was performed using MetaMorph software normalizing actin intensity over a 30-mm area to Syto 60 staining (B). Means values ± SEM are shown from 3 independent experiments with 4-6 images collected per experiment. *, $P<0.05$ versus control.

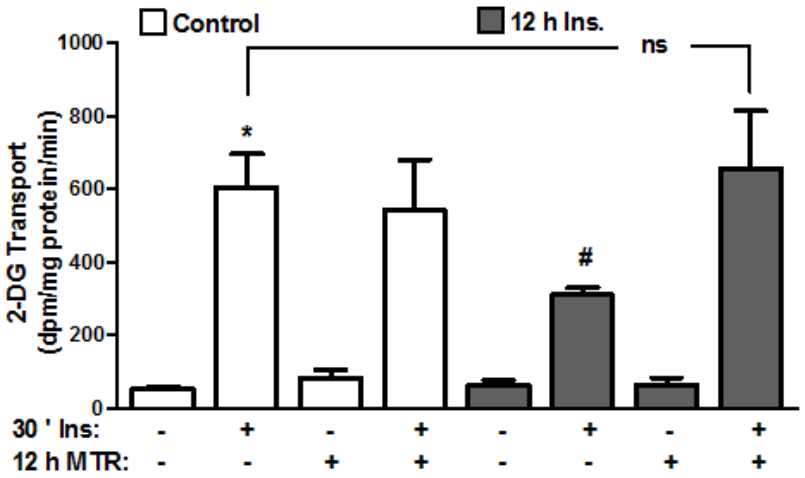

Figure 12. Inhibition of Sp1 binding activity protects against glucose transport dysfunction. 3T3-L1 adipocytes were pretreated with or without 100 nM mithramycin (MTR) for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, cells were placed in serum free media for 1 h. During the last 30 min, cells were incubated with 100 nM insulin to stimulate glucose uptake. Means values ± SEM are shown from 5 independent experiments. *, $P<0.05$ versus unstimulated control. #, $P<0.05$ versus stimulated control.

activation of the HBP previously shown (7), activation of this pathway promotes an increase in *O*-GlcNAc on Sp1 (**Fig. 13**). Second, this increase in *O*-GlcNAc promotes an increase in the binding affinity of this transcription factor toward the promoter regions of cholesterolgenic genes. Third, we provide evidence that the HBP plays a direct role in the activation of the promoter region of HMGR as hyperinsulinemia promoted and HBP inhibition protected against increases in luciferase reporter activity. Fourth, it was found that *O*-GlcNAc of Sp1 may play a crucial role in the activation of a cholesterolgenic program that promotes insulin resistance. Inhibition of Sp1 with mithramycin resulted in a restoration of protein levels of HMGR. Concurrently, mithramycin treatment also protected against PM cholesterol accrual, F-actin loss, and impaired glucose transport induced by hyperinsulinemia. In continuation of these studies, we next aimed to determine the role of the micronutrient chromium in attenuating glucose flux through this pathway. Of particular interest is whether an effect of chromium on this pathway could serve as a basis of its action in benefiting glucose and lipid metabolism.

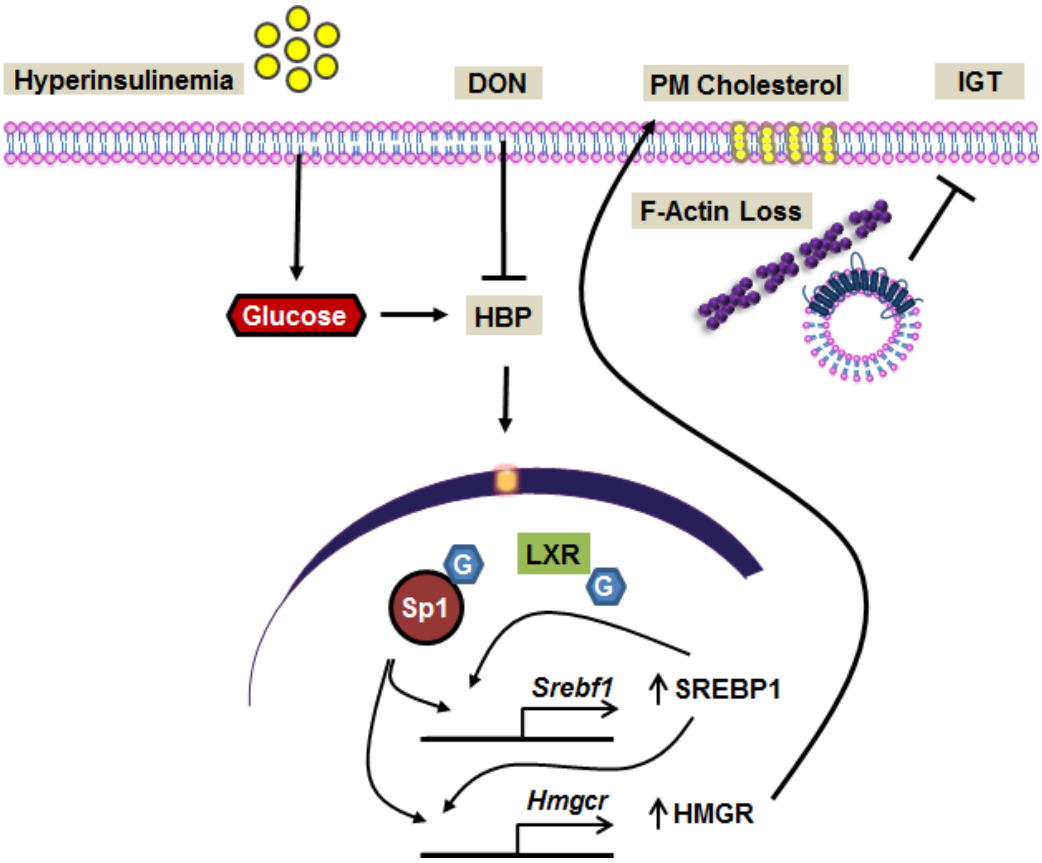

Figure 13. Model of hyperinsulinemia-induced insulin resistance in adipocytes. Exposure of 3T3-L1 adipocytes to hyperinsulinemia induces insulin resistance. Specifically, exposure to hyperinsulinemia promotes an increase in *O*-GlcNAc on Sp1, leading to its increased binding to the promoter regions of the genes *Srebf1* and *Hmgcr*. Increases in the protein product SREBP1 can positively feedback resulting in activation of its own promoter as well as the promoter of HMGR. This could also occur through the saturation of Insig2, leading to increased processing of SREBP1. The resulting increases in HMGR promote an accumulation of PM cholesterol, disruption of F-actin structure, and impairment in glucose transport (IGT). Treatment with the HBP inhibitor DON or the Sp1 binding inhibitor mithramycin (not shown) protects against PM

cholesterol accumulation and defects in insulin responsiveness. Other transcription factors, such as LXR, are known to be targets of the HBP and may combinatorially promote the activation of genes involved in regulating cholesterol synthesis, thereby impairing glucose transport.

II.B. Chromium Improves Cellular Cholesterol Efflux, ABCA1 Functionality, and Rab8 Cycling Rendered Defective by Hyperinsulinemia in Adipocytes

II.B.1. Summary

Studies presented in the previous chapter suggested a novel mechanism by which hyperinsulinemia could promote alterations in cellular cholesterol homeostasis through engaging the HBP. A puzzling observation, however, presented itself in that previous *in vivo* analyses validated elevations in membrane content associated with insulin resistance (11, 338). This finding was paradoxical in light of the fact that the RCT should protect against increases in PM cholesterol content through efflux processes. This would result in the elimination of excess cholesterol to a growing HDL particle, which would then be transported to the liver for secretion in the bile. Given this observation, the studies presented in this chapter sought to discern the mechanisms whereby insulin resistance, induced by hyperinsulinemia, could promote alterations in cholesterol efflux in 3T3-L1 adipocytes. Concurrently, these studies sought to elucidate a mechanistic understanding for the role of chromium in promoting optimal lipid metabolism. The generation of the rate-limiting HDL sub-particle, pre-β-1, was found to be impaired in adipocytes exposed to hyperinsulinemia and corrected with concurrent culturing in CrPic. Mechanistically, it was found that endosomal cholesterol content was elevated by hyperinsulinemia and reversed with CrPic. This increase and restoration of endosomal cholesterol levels was associated with an impairment and correction of ABCA1 trafficking to the PM,

respectively. Furthermore, it was found that the cycling of Rab8, known to be found on the same vesicles as ABCA1, was impaired with hyperinsulinemia and corrected with CrPic treatment. Finally, it was observed that inhibition of the HBP or direct activation of AMPK mimicked the effects of CrPic on improving these parameters and cholesterol efflux processes. Taken together, this data suggests an unappreciated basis for the coexistence of low HDL and metabolic derangements such as hyperinsulinemia. This data also places increased HBP activity as a potential mediator of defects in both glucose and cholesterol metabolism.

II.B.2. Results

Hyperinsulinemia Impairs ApoA-I-mediated Cholesterol Efflux in Adipocytes: Previous studies conducted in humans with similar levels of total HDL showed marked differences in the ability of macrophages to efflux cholesterol, a cardioprotective event. In delineating the cause, it was determined that the ability of macrophages to efflux cholesterol in serum with similar levels of HDL correlated with the concentration of pre-β-1 HDL in the serum (339). While several tissues contribute to this sub-fraction, recent evidence seeking to define the relationship between insulin resistance and low HDL has suggested that the expanding pool of cholesterol found in adipose tissue may play an important role in these processes *in vivo* (201, 340). As such, studies first attempted to discern the effects of hyperinsulinemia on the generation of pre-β-1 HDL. Treatment of 3T3-L1 adipocytes with hyperinsulinemia (5 nM, 12 h) resulted in an

approximately 30% impairment in the ability of these cells to efflux cholesterol compared to control cells (**Fig. 14**). Strikingly, treatment with CrPic prevented against the impairment in cholesterol efflux induced by hyperinsulinemia. To determine if the alterations in cholesterol efflux resulted from a loss in the protein content of ABCA1, total levels were examined in these conditions. Interestingly, total levels of ABCA1 were found to be unchanged, yet trended (P=0.20) toward being increased in cells cultured with hyperinsulinemia, suggesting a loss of total ABCA1 could not account for the impaired efflux (**Fig. 15A**). It was thus next examined if perhaps the trafficking of ABCA1 to the PM was affected by hyperinsulinemia. Treatment with hyperinsulinemia was found to provoke an approximate 40% loss in the amount of ABCA1 present at the PM (**Fig. 15B**). CrPic treatment restored PM ABCA1 to levels witnessed in control cells. Reciprocally, an almost 3 fold increase in endosomal ABCA1 was detected in cells treated with hyperinsulinemia (**Fig. 15C**). This increase in endosomal ABCA1 was normalized upon treatment with CrPic. Together this suggested a defect in delivery of ABCA1 to the PM as a basis for the impaired cholesterol efflux.

Hyperinsulinemia Impairs the Functional Cycling of Rab8: Recent findings have established that the small G-protein Rab8 serves as a key protein in the regulatory machinery leading to the redistribution of ABCA1 to the PM to facilitate the removal of cholesterol (218, 219). Furthermore, it has been shown that in conditions such as NPC, alterations in cholesterol content can sequester Rab proteins on endosomal membranes, thereby keeping them in an inactive

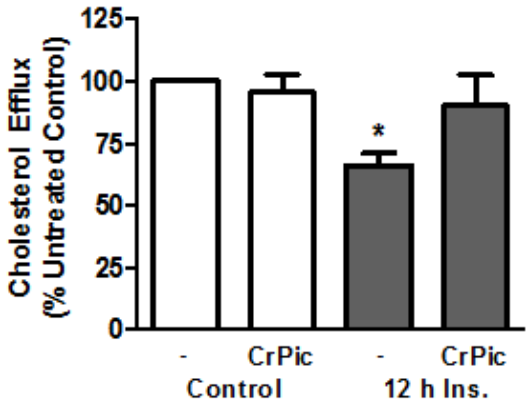

Figure 14. Hyperinsulinemia impairs cholesterol efflux in adipocytes. 3T3-L1 adipocytes were labeled with 0.5 µCi/ml ^{3}H-cholesterol for 24 h. Cells were then washed and pretreated with or without 1 µM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. Following treatments, cells were placed in media containing 0.2% BSA and 10 µg/ml lipid-free ApoA1 for 4 hours. ^{3}H-cholesterol was then measured in the medium and the cells by using a scintillation counter. The percentage of acceptor-specific efflux was calculated using the equation: medium/(medium+cells). Values obtained in the absence of acceptor were subtracted to account for non-specific ^{3}H-cholesterol efflux/leakage. Means values ± SEM are shown from 3-10 independent experiments. *, P<0.05 control.

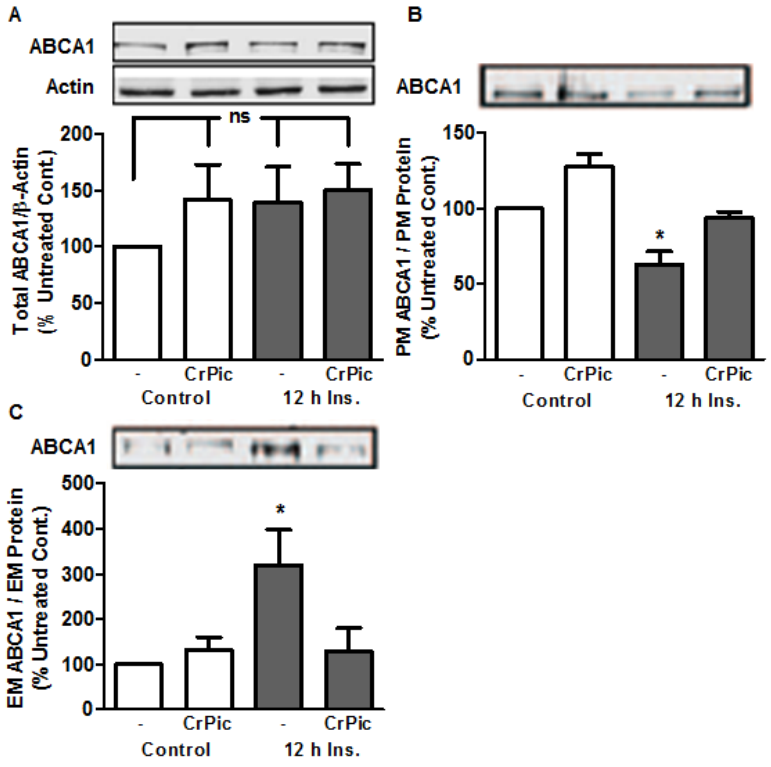

Figure 15. Hyperinsulinemia impairs ABCA1 trafficking to the PM. 3T3-L1 adipocytes were pretreated with or without 1 µM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. Following treatments, whole cell lysates were prepared and protein levels of ABCA1 were determined by immunoblotting (A), normalized to actin. Mean values ± SEM are shown from 3-4 independent experiments. PM fractions were prepared and ABCA1 protein levels were determined by immunoblotting (B), normalized to Ponceau. Mean values ± SEM are shown from 4-8 independent experiments. *, $P<0.05$ control. Endosomal membrane (EM) fractions were prepared and ABCA1 protein levels were determined by immunoblotting (C), normalized to Ponceau. Mean values ± SEM are shown from 4-7 independent experiments. *, $P<0.05$ control.

form on membranes and preventing their cycling to the cytosol to allow for dynamic and continued trafficking (341). As such, it was next examined whether the disrupted delivery of ABCA1 to the PM could be due to alterations in the functional cycling of Rab8. Similar to ABCA1, Rab8 was increased by approximately 50% in endosomal fractions of cells treated with hyperinsulinemia (**Fig. 16A**). This increase was prevented in cells that were concurrently treated with CrPic. Concomitant with this increase in endosomal Rab8 content was a diminished level of Rab8 found in cytosolic fractions obtained from cells treated with hyperinsulinemia (**Fig. 16B**). Treatment with CrPic in the presence of hyperinsulinemia prevented this approximately 30% loss in cytosolic Rab8 content. Taken together, this data suggested that the loss in ABCA1 delivery to the PM could be the consequence of alterations in the appropriate cycling of this protein.

Hyperinsulinemia Promotes and AMPK Activation Attenuates Increased Endosomal Cholesterol Content: To determine if increased endosomal cholesterol content could be mediating the dysfunctional ABCA1 trafficking and Rab8 cycling, this parameter was next examined. Cholesterol analyses revealed a substantial increase in endosomal cholesterol content in cells cultivated under hyperinsulinemic conditions (**Fig. 17A**). This increase in cholesterol content was corrected upon treatment with CrPic. Interestingly, recent evidence suggests that AMPK might be a target of chromium action, suggesting a potential mechanism for the beneficial effect of CrPic treatment on endosomal cholesterol (281). As such, it was next examined if CrPic resulted in an activation of AMPK in these

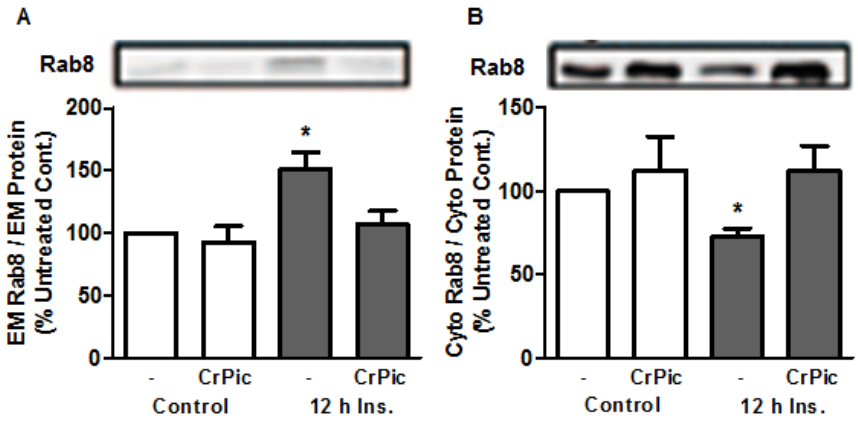

Figure 16. Hyperinsulinemia impairs the functional cycling of Rab8. 3T3-L1 adipocytes were pretreated with or without 1 µM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. Following treatments, endosomal membrane (EM) fractions were prepared and protein levels of Rab8 were determined by immunoblotting (A), normalized to Ponceau. Mean values ± SEM are shown from 5-13 independent experiments. *, $P<0.05$ control. Cytosolic fractions were also prepared and protein levels of Rab8 were determined by immunoblotting (B), normalized to Ponceau. Mean values ± SEM are shown from 5-13 independent experiments. *, $P<0.05$ control.

cells. These studies revealed that treatment with CrPic resulted in an activation of AMPK in both control and hyperinsulinemic cells (**Fig. 17B**). In addition, the activation observed was found to be similar to that induced by the AMPK activator, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboximide (AICAR). In fact, AICAR was also found to restore endosomal membrane cholesterol content to levels observed in control cells (**Fig. 17C**). This treatment was also found to correct the dysfunctional ABCA1 trafficking and Rab8 cycling induced by hyperinsulinemia.

Inhibition of the HBP or Activation of AMPK Restores Cholesterol Efflux: In contrast to AMPK, increases in HBP flux have been shown in the previous section to provoke cholesterol accrual induced by hyperinsulinemia. The effect of HBP inhibition and activation of AMPK by AICAR on cholesterol efflux was next examined. These studies revealed that DON and AICAR had similar effects as CrPic with regard to preventing the impairment in cholesterol efflux induced by hyperinsulinemia (**Fig. 18**). Neither agent, nor CrPic, displayed any effects on efflux processes in control cells. In addition, the cholesterol lowering agent, methyl-β-cyclodextrin, was also observed to correct against defective cholesterol efflux.

In summary, data from these *in vitro* studies presented in Chapter IIB offer several novel observations. First, they add growing support for the role of adipocytes in cholesterol efflux with data suggesting that this process becomes impaired by pathophysiological hyperinsulinemia. Second, they suggest that hyperinsulinemia-induced cholesterol accrual is not simply the result of ectopic

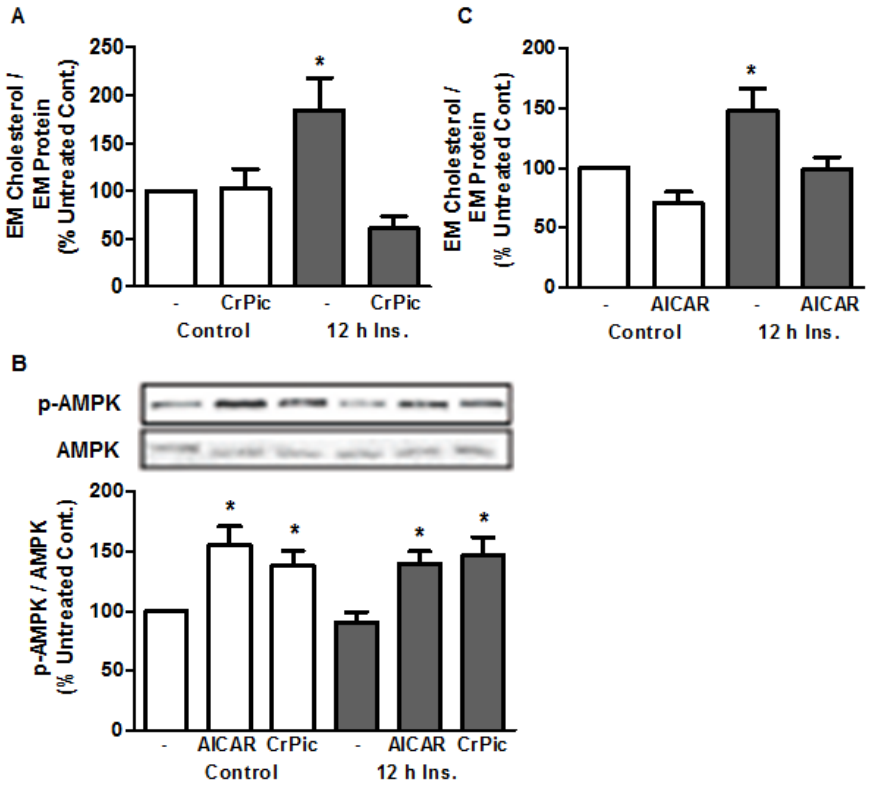

Figure 17. Hyperinsulinemia-induced endosomal cholesterol accrual is mitigated with CrPic or AICAR. 3T3-L1 adipocytes were pretreated with or without 1 µM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. AICAR treatments were performed after the 12 h insulin exposure for 45 min. Following treatments, endosomal membrane (EM) fractions were prepared and cholesterol content was measured (A and C) normalized to total endosomal protein content. Mean values ± SEM are shown from 3-8 independent experiments. *, $P<0.05$ control. Following treatments, whole cell lysates were also prepared and phosphorylation of AMPK was determined by immunoblotting (B), normalized to total levels of AMPK. Mean values ± SEM are shown from 6 independent experiments. *, $P<0.05$ control.

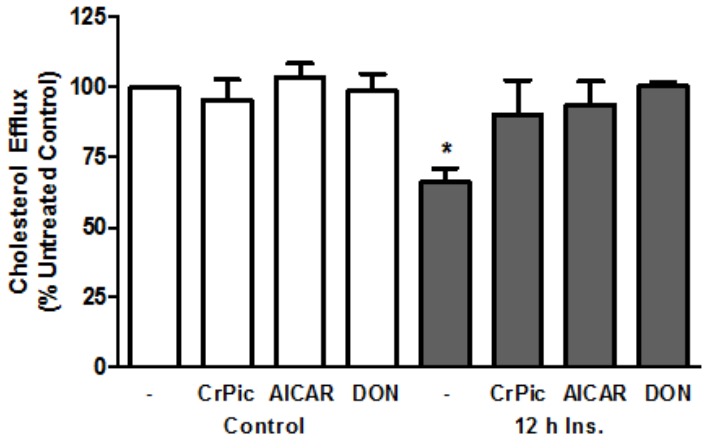

Figure 18. AMPK activation and HBP inhibition protect against hyperinsulinemia-induced impairments in cholesterol efflux. 3T3-L1 adipocytes were labeled with 0.5 µCi/ml ^{3}H-cholesterol for 24 h. Cells were then washed and pretreated with or without 1 µM CrPic or 20 µM DON for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. AICAR treatments were performed after the 12 h insulin exposure for 45 min. Following treatments, cells were placed in media containing 0.2% BSA and 10 µg/ml lipid-free ApoA1 for 4 hours. ^{3}H-cholesterol was then measured in the medium and the cells by using a scintillation counter. The percentage of acceptor-specific efflux was calculated using the equation: medium/(medium+cells). Values obtained in the absence of acceptor were subtracted to account for non-specific ^{3}H-cholesterol efflux/leakage. Means values ± SEM are shown from 3-10 independent experiments. *, P<0.05 control.

cholesterol at the PM as endosomal cholesterol content was also found to be elevated. Third, these data support a mechanism by which endosomal cholesterol accumulation induced by hyperinsulinemia could stabilize Rab8 on these membranes, thereby interfering with the necessary cycling of this protein for optimal delivery of ABCA1 to the PM (**Fig. 19**). These data also suggest that activation of AMPK by CrPic or AICAR could improve cholesterol efflux processes by inhibiting cholesterol synthesis and accrual in the endosomal membranes. Further, inhibition of the HBP mimicked these beneficial effects, suggesting a role of this pathway in promoting derangements in lipoprotein metabolism in addition to glucose homeostasis. Of particular interest for future studies was to determine if CrPic treatment, perhaps through activation of AMPK, could impinge upon the HBP pathway to exert beneficial effects on glucose metabolism in adipose tissue and skeletal muscle.

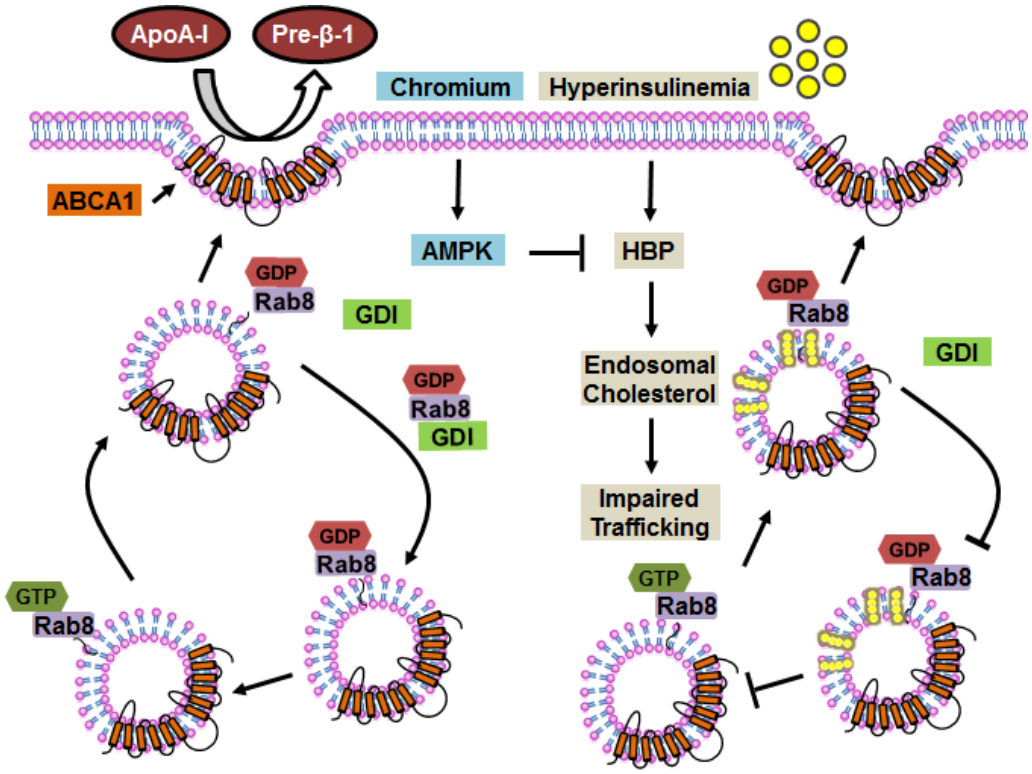

Figure 19. Model of hyperinsulinemia-induced impairment in cholesterol efflux. Under healthy conditions, 3T3-L1 adipocytes respond to cell signaling processes to promote removal of excess cholesterol or phospholipids to lipid poor ApoA-I. This process is mediated by Rab8, which delivers ABCA1 to the PM, is extracted by GDI, and is redelivered to endosomal pools to promote dynamic trafficking of ABCA1 to the PM. In adipocytes, chronic exposure to hyperinsulinemia results in the activation of the HBP, promoting excess PM and EM cholesterol content. These increases in EM cholesterol content may perturb the ability of Rab8 to be extracted by GDI, thereby sequestering it in endosomal pools and inhibiting the proper delivery of ABCA1 to the PM. Treatment of adipocytes with chromium resulted in the activation of AMPK. This increase in AMPK activity was associated with a prevention of hyperinsulinemia-induced EM cholesterol accrual as well as dysfunctional trafficking of ABC1 to the PM. AMPK

stimulation by AICAR (not shown) also promoted the appropriated cycling and trafficking of Rab8 and ABCA1, respectively, correcting impaired cholesterol efflux. Additionally, direct inhibition of the HBP was found to protect against these derangements and restore cholesterol efflux.

II.C. Chromium Protects Against Hexosamine-Induced Cholesterol Accumulation and Insulin Resistance

II.C.1. Summary

Studies presented in the previous chapters suggested a novel mechanism by which hyperinsulinemia could engage the HBP, potentially promoting alterations in both PM and EM cholesterol content. Direct inhibition of the HBP prevented glucose transport dysfunction as well as perturbed trafficking of ABCA1 to the PM. In addition, it was observed that chromium had similar effects as HBP inhibition with regard to cholesterol efflux studies. Given the numerous studies demonstrating a beneficial effect of chromium supplementation on at least one parameter of glucose metabolism in humans (23, 233, 234, 236-238, 240), we sought to determine if chromium could impinge on the HBP, thereby promoting an attenuation in PM cholesterol accrual and impaired glucose uptake. In adipocytes treated with hyperinsulinemia, a characteristic increase in the protein/mRNA levels of HMGR was observed. Additionally, increases in the *O*-GlcNAc status of Sp1 were found under these conditions, associated with similar gains in promoter affinity and activity with regard to HMGR. This was associated with an impairment in insulin action despite intact insulin signaling to AS160. Strikingly, CrPic resulted in an attenuation of HBP pathway activity as assessed by glycosylation of Sp1. In congruence with the effect of glycosylation in promoting an increased affinity to HMGR, CrPic resulted in a reduction in the affinity to the HMGR promoter as well as promoter activity as assessed by ChIP and luciferase assays. While hyperinsulinemia promoted an impairment in insulin

responsiveness, concurrent treatment with CrPic prevented this effect. CrPic treatment did not amplify insulin signaling to mediate the improved responsiveness. *In vivo* validations studies were performed in C57Bl/6J mice placed on an 8 week low-/high-fat diet with or without CrPic supplementation. Mice fed a high-fat diet displayed glucose intolerance and increased circulating plasma insulin compared to low-fat fed littermates. In addition, epididymal fat pads taken from these mice displayed increases in cholesterolgenic processes and membrane cholesterol content. In high-fat animals supplemented with CrPic these cellular derangements were prevented and insulin sensitivity was markedly improved.

II.C.2. Results

CrPic Protects Against Hyperinsulinemia-induced PM Cholesterol Accrual: Results presented in the previous chapter demonstrate that pathophysiologic hyperinsulinemia promotes increases in PM cholesterol content, in turn perturbing the cortical F-actin structure necessary for proper GLUT4 translocation. *Ex vivo* examination of skeletal muscle tissues from insulin-resistant Zucker rats demonstrated that correction of membrane cholesterol restores F-actin structure and insulin sensitivity (338). Studies first evaluated whether CrPic treatment could protect against hyperinsulinemia-induced PM cholesterol accrual. PM cholesterol content was elevated by approximately 50% the gain in membrane cholesterol content observed with hyperinsulinemia. No alterations in cholesterol content were observed in control cells treated with

CrPic. To determine if CrPic treatment was correcting cholesterol content through an inhibition of synthesis, total levels of HMGR were analyzed. Under hyperinsulinemic conditions, HMGR was found to be increased by 60% (**Fig. 20B**). CrPic treatment inhibited this effect. To next determine if the alterations in the protein levels of HMGR were the result of alterations in transcription, mRNA levels of HMGR were assessed. These analyses revealed that hyperinsulinemia promoted an approximate 5 fold increase in the mRNA expression of HMGR that CrPic corrected (**Fig. 20C**). No effect of CrPic treatment on HMGR protein or mRNA content was observed in control cells.

CrPic Reduces HBP-induced *O*-GlcNAc Modification of Sp1: Work presented in the previous chapters linked excessive glucose flux through the HBP in provoking a cholesterolgenic response through the activation of Sp1 by *O*-GlcNAc. To test where or not CrPic could inhibit the increased HMGR mRNA/ protein through altering glucose flux through the HBP and subsequent modification of Sp1, immunoprecipitation was performed. These analyses revealed an approximate 55% increase in the *O*-GlcNAc modification of Sp1 induced by hyperinsulinemia (**Fig. 21**). While CrPic treatment did not affect this parameter in control cells, treatment blunted the effects of hyperinsulinemia in engaging this pathway. Together, these results suggested inhibition of HBP-induced cholesterol synthesis as a potential mechanism for CrPic action to improve insulin responsiveness.

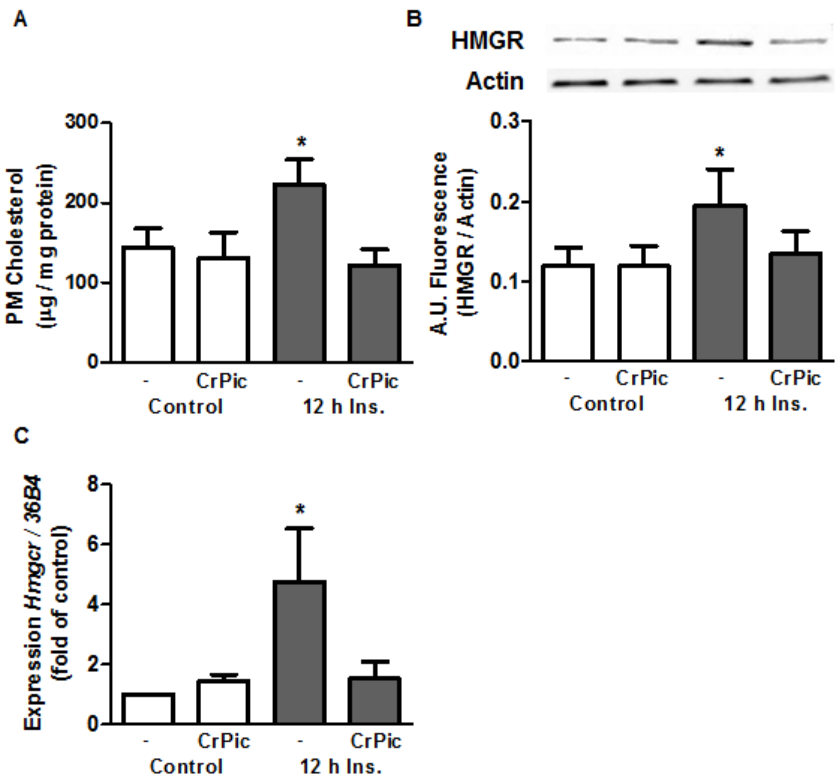

Figure 20. CrPic protects against hyperinsulinemia-induced cholesterol synthesis. 3T3-L1 adipocytes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. Following treatments, PM cholesterol content (A) was assessed and normalized to total PM protein content. Mean values ± SEM are shown from 5 independent experiments. *, P<0.05 versus control. Protein levels of HMGR (B) were determined by immunoblotting, normalized to actin. Mean values ± SEM are shown from 4 independent experiments. *, P<0.05 versus control. The mRNA content of *Hmgcr* (C) was also determined and normalized to *36B4*. Means values ± SEM are shown from 5 independent experiments. *, P<0.05 versus control.

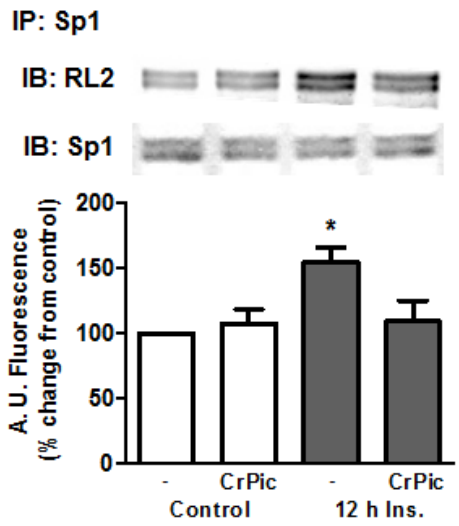

Figure 21. Hyperinsulinemia provokes and CrPic inhibits *O*-linked glycosylation of Sp1. 3T3-L1 adipocytes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, lysates were prepared and immunoprecipitated with an Sp1 antibody. Eluted samples were then immunobloted with an RL2 antibody to detect *O*-linked glycosylation of Sp1. The glycosylation of Sp1 was normalized to an input sample of Sp1 from lysed samples. Means values ± SEM are shown from 5-6 independent experiments. *, $P<0.05$ versus control.

Hyperinsulinemia Augments and CrPic Attenuates Sp1 Promoter Affinity: Study next sought to determine if CrPic treatment, potentially through reducing *O*-GlcNAc modification of Sp1, could alter its binding affinity toward the promoter region of HMGR. This could be mediated through direct targeting of Sp1 toward the promoter region or alternatively, through preventing the degradation of this protein as study has previously demonstrated (134, 202). To further examine this process, ChIP was performed. Hyperinsulinemic culturing conditions resulted in a characteristic increase in the association of Sp1 toward the promoter (**Fig. 22**), whereas concurrent treatment with CrPic markedly attenuated this association.

CrPic Protects against Hyperinsulinemia-induced Activation of the HMGR Promoter: Study next sought to delineate the effects of CrPic treatment on the transcriptional activity of the HMGR promoter. Plasmids containing the coding sequence (-284 to +36) of the promoter, containing 3 Sp1 binding moieties, were electroporated into adipocytes. Further *in silico* analyses conducted using MatInspector demonstrated the consensus binding sites for Sp1 as well as other transcription factors involved in the activation of HMGR (**Fig. 23A**). Next, luciferase assays were performed to examine how CrPic treatment affected the promoter activity. In cells exposed to hyperinsulinemia, a 2 fold increase in the promoter activity was observed (**Fig. 23B**). Consistent with CrPic inhibiting this response through reducing the affinity of Sp1 to the promoter, this treatment blunted the increase in activity observed with hyperinsulinemia. Of note, CrPic did not have any effect on the luciferase activity in control cells.

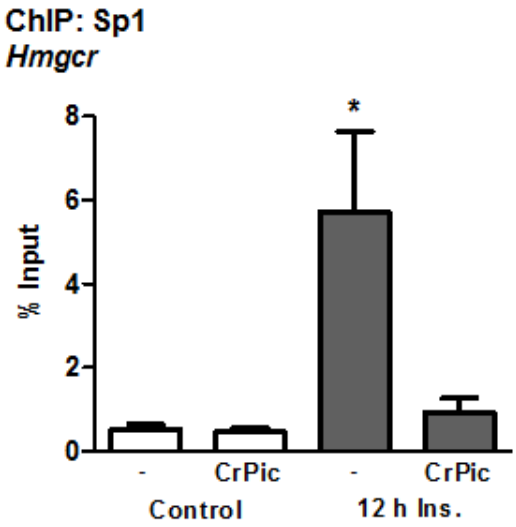

Figure 22. CrPic reduces hyperinsulinemia-induced association of Sp1 toward the HMGR promoter. 3T3-L1 adipocytes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, DNA was sonicated and chromatin immunoprecipitation was performed using an Sp1 antibody. Purified DNA and primers specific to the Sp1 binding site in the promoter region of HMGR were utilized for qPCR analyses. Ct values from qPCR were normalized using the percent input method. Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

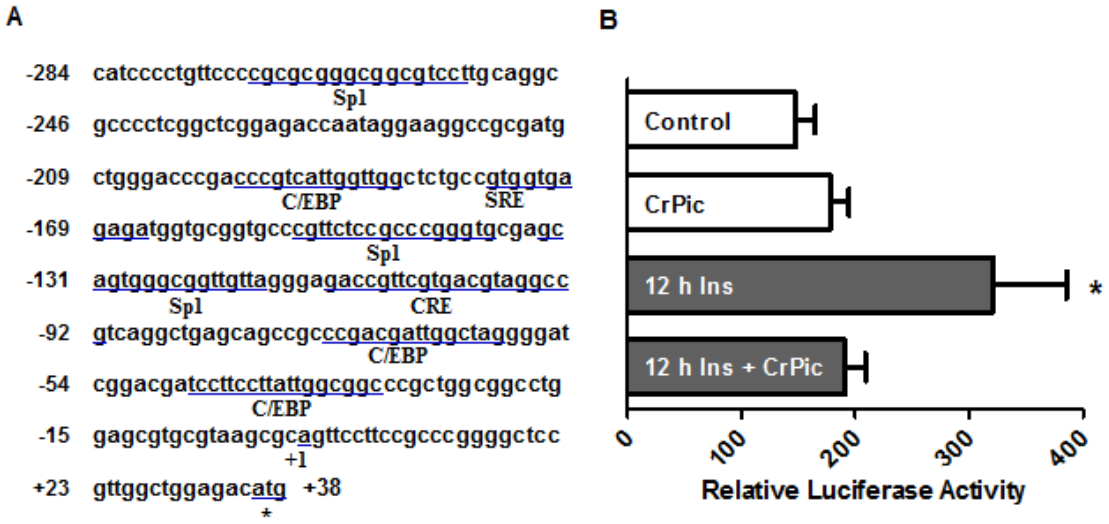

Figure 23. Hyperinsulinemia activates and CrPic reduces promoter activity of HMGR. 3T3-L1 adipocytes were electroporated with the minimal promoter sequence (-284 to +36) of HMGR cloned into pGL2B luciferase reporter plasmids (A). The location of various sequence motifs that serve as sites of recognition for transcription factors are underlined. The +1 indicates the start site of transcription and the asterisk indicates the start site of translation. After a 16-18 h recovery period, cells were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, luciferase activity was measured (B) and normalized relative to Renilla. Means values ± SEM are shown from 3 independent experiments. *, $P<0.05$ versus control.

CrPic Protects against Impaired Glucose Transport: With data suggesting that CrPic could protect against hyperinsulinemia-induced defects in insulin responsiveness through attenuation of cholesterol synthesis, glucose uptakes were next performed. Insulin-stimulated glucose transport was found to be reduced by approximately 55% with hyperinsulinemia (**Fig. 24**). CrPic treatment completely ameliorated the glucose transport dysfunction induced by hyperinsulinemia. Studies have suggested a beneficial mechanism of CrPic action on glucose transport might entail countering defects in insulin signaling observed in animal models of insulin resistance (342-345). Yet other lines of evidence suggest that pathophysiologic hyperinsulinemia (5 nM) does not impair insulin signaling (8, 12). It was thus next characterized whether the 500 pM dose of insulin induced a defect in insulin signaling and, if so, whether CrPic treatment could counter this effect. Hyperinsulinemia, however, was not found to alter insulin-stimulated phosphorylation of Akt2 at Ser 474 (**Fig. 25A**), nor did it affect the phosphorylation of its downstream substrate, AS160 (**Fig. 25B**). Similarly, CrPic treatment had no effect on insulin signaling in control cells, nor did it have a beneficial action in cells co-treated with hyperinsulinemia.

***In Vivo* CrPic Supplementation Study Design:** Taken together, these *in vitro* findings suggested that CrPic may benefit glucose metabolism by inhibiting HBP activity thereby attenuating cholesterol synthesis. To begin to translate these findings, study utilized the obesity-prone C57Bl/6J mice placed on a LF or HF diet over a period of 8 weeks. The development of obesity and insulin resistance as a result of excessive intake of dietary fat has been established in

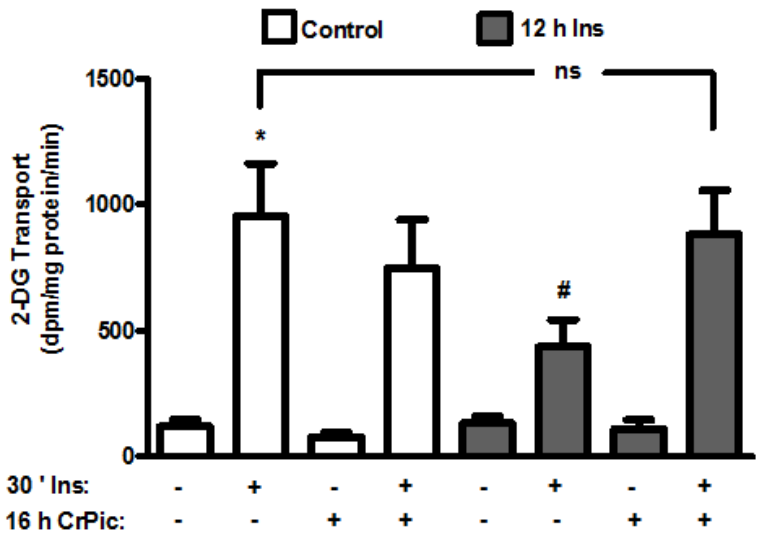

Figure 24. CrPic attenuated glucose transport dysfunction induced by hyperinsulinemia. 3T3-L1 adipocytes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, cells were placed in serum free media for 1 h. During the last 30 min, cells were incubated with 100 nM insulin to stimulate glucose uptake. Means values ± SEM are shown from 5 independent experiments. *, P<0.05 versus unstimulated control. #, P<0.05 versus stimulated control.

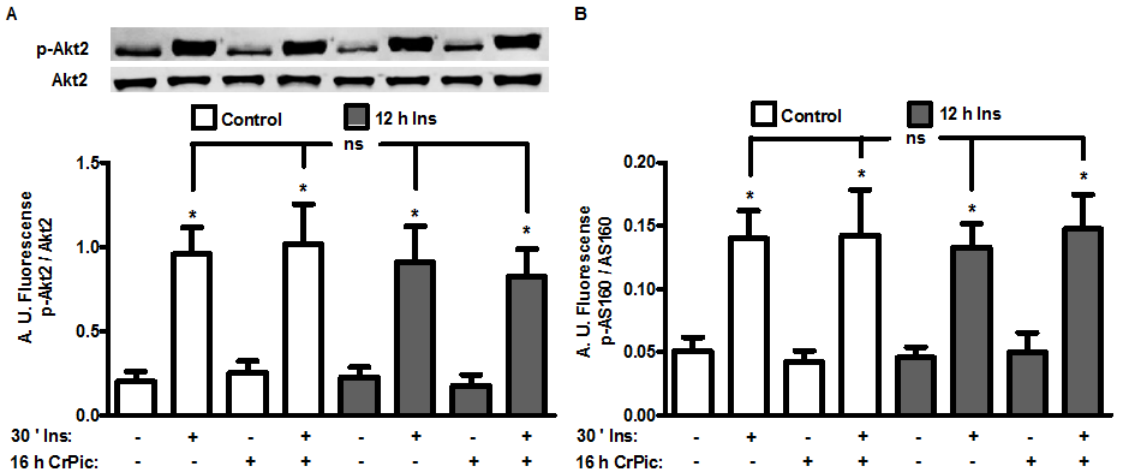

Figure 25. CrPic did not enhance nor did hyperinsulinemia impair insulin signaling. 3T3-L1 adipocytes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 500 pM insulin for 12 h. Following treatments, cells were placed in serum free media for 1 h. During the last 5 min, cells were incubated with 100 nM insulin to induce phosphorylation of insulin signaling proteins. Phosphorylation of Akt (A) was determined by immunoblotting, normalized to total Akt. Means values ± SEM are shown from 4 independent experiments. *, P<0.05 versus respective unstimulated treatment. Phosphorylation of AS160 (B) was also determined by immunoblotting, normalized to total AS160. Means values ± SEM are shown from 4 independent experiments *, P<0.05 versus respective unstimulated treatment.

these animals by several studies (346-351), For these studies, chromium supplementation was dissolved in the drinking water at a dose of 8 µg/kg body weight per day, which given an average human body mass of 75 kg, represents a clinically efficacious dose of 600 µg. The mice in these studies were fed *ad libitum* a NIH standard chow diet for two weeks and then subsequently placed on a modified LF diet containing palm oil instead of lard to better mimic the fatty acid composition of western societies (352). After a three week acclimation period, mice were divided into one of four groups (**Fig. 26**). LF fed mice were given 20% kcal from protein, 70% kcal from carbohydrates, and 10% kcal from fat. In contrast, the HF diet consisted of 20% kcal from protein, 35% kcal from carbohydrates and 45% kcal from fat. In addition, the diets were made with a chromium free mineral mix (S17902) and Avicel PH101 (instead of cellulose, which contains chromium). Chromium potassium sulfate was then added back to the diets to provide a 380 µg, which given a 0.5% absorption, represents approximately 2 µg in the bloodstream. Body weight was measured once per week throughout the course of the study and was found to be significantly elevated by HF feeding (**Fig. 27**). While chromium supplementation has garnered popularity by the belief that it can mitigate weight gain, study has demonstrated that doses up to 1000 µg do not independently influence body weight (353, 354). In congruence with these data, CrPic did not affect weight gain in mice fed a HF diet. Importantly, the observed changes in body weight were independent of changes in food consumption at any point in the study (**Fig. 28A**). Additionally, water consumption was measured once per week throughout the study,

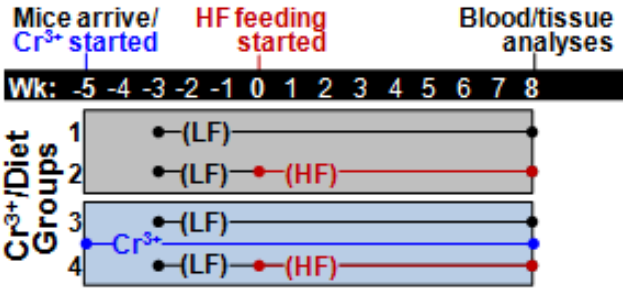

Figure 26. Study design for diet intervention and CrPic supplementation. C57Bl/6J mice 4 weeks of age were acclimated with or without CrPic supplementation in their drinking water. Mice were fed *ad libitum* NIH standard chow for a period of 2 weeks. After this time, mice were placed on the modified LF diet for 3 additional weeks. At time 0, mice were then placed on either a LF or HF diet with or without CrPic supplementation for a period of 8 weeks. All procedures and *ex vivo* tissue analyses were performed at the completion of this dietary intervention.

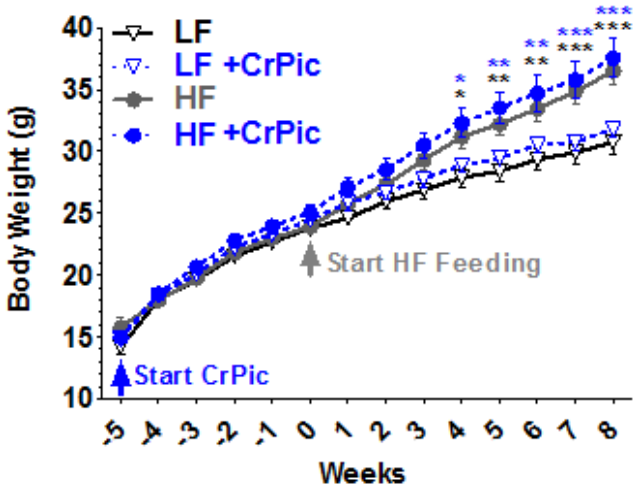

Figure 27. HF feeding regardless of chromium status increases body weight. C57Bl/6J mice were initially acclimated on a LF diet in the presence (blue) or absence (black) of chromium in their drinking water. At 0 weeks, mice were then separated again into LF (triangle) or HF (circle) groups ± continued supplementation with chromium. Body weight was measured once per week throughout the duration of the study. Means values ± SEM are shown from 12 mice per group. *, P<0.05 versus LF. **, P<0.01 versus LF. ***, P<0.001 versus LF.

averaging 2.6-2.7 ml/day (**Fig. 28B**). Careful monitoring of this intake was necessary to ensure appropriate CrPic supplementation.

CrPic Improves Glucose and Insulin Tolerance in Mice Fed a HF Diet: To characterize the effect of HF feeding and CrPic supplementation on whole body glucose tolerance and insulin sensitivity, intraperitoneal glucose and insulin tolerance tests (IPGTT and IPITT) were performed on C57Bl/6J mice following dietary intervention. For these analyses, mice were fasted for 6 hours prior to testing, as previous study has demonstrated this fasting interval is best for assessing glucose tolerance (355). In the glucose tolerance testing, mice were injected with 2 g D-glucose/kg body weight and for IPITT mice were injected with 1.0 unit of insulin/kg body weight. Mice fed a HF diet had a marked impairment in glucose tolerance compared to LF fed mice (**Fig. 29A**). Mice fed a HF diet supplemented with CrPic had an improvement in glucose tolerance. Area under the curve for the glucose tolerance test also demonstrated an impairment with HF feeding and an improvement with CrPic supplementation (**Fig. 29B**). To supplement the glucose tolerance data, insulin tolerance testing was performed in these mice after 8 weeks of HF feeding. In LF fed mice, insulin promoted a rapid reduction in plasma glucose (**Fig. 30A**). In contrast, the ability of insulin to lower plasma glucose levels over the course of the study was reduced in HF fed mice, while CrPic supplementation improved insulin sensitivity. The area above the curve for the insulin tolerance test further evinced this alteration in insulin sensitivity (**Fig. 30B**). As a final measure of insulin sensitivity, random fed plasma insulin concentrations were also assessed in these mice for evidence of

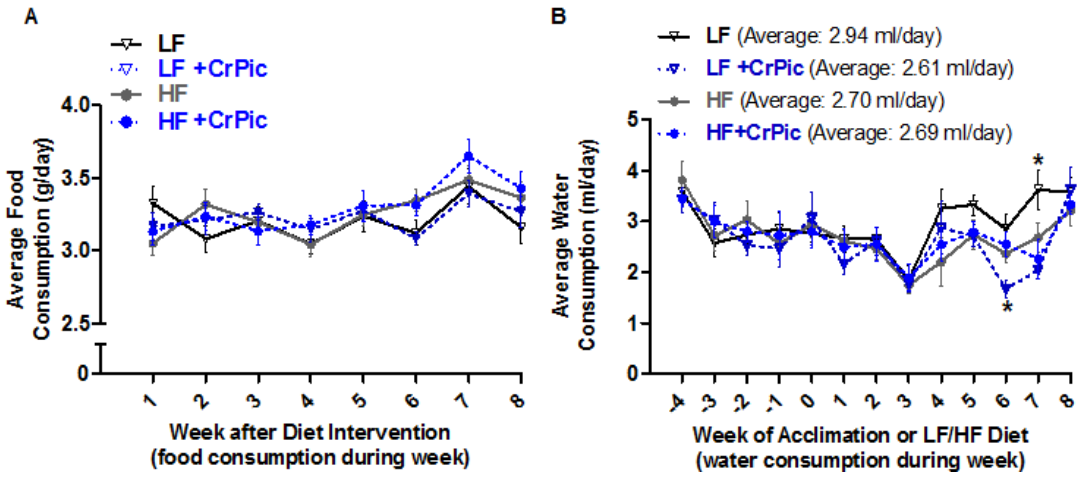

Figure 28. HF feeding and CrPic supplementation did not affect food or water consumption. Food (A) and water (B) consumption was measured once per week throughout the study. Weekly values obtained were divided by the number of days between each determination to obtain average food or water consumption by day values. Means values ± SEM are shown from 12 mice per group. While HF feeding and CrPic did not affect water consumption throughout most of the study, a significant difference was observed in the LF + CrPic group at 6 weeks post dietary intervention and in the LF - CrPic group at 7 weeks post dietary intervention. *, $P<0.05$ versus all other groups.

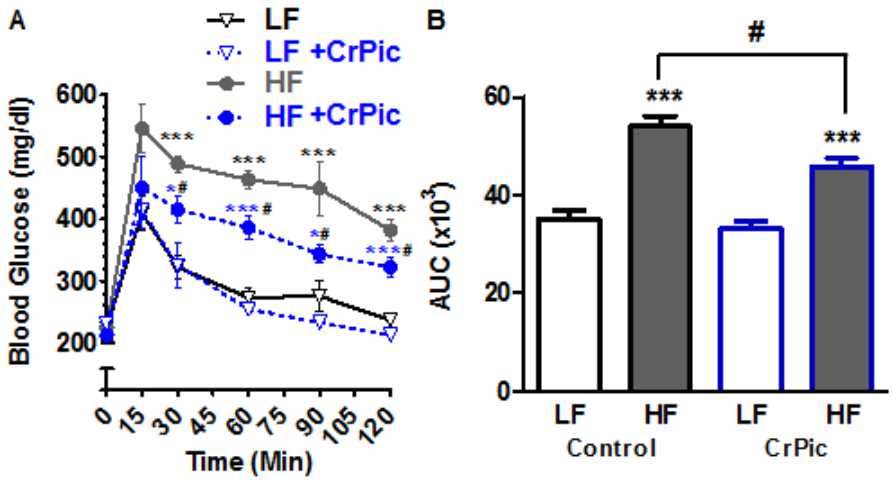

Figure 29. HF feeding promotes an impairment and CrPic supplementation improves glucose tolerance in C57Bl/6J mice. Intraperitoneal glucose tolerance tests were performed on mice following 8 weeks of dietary intervention. Mice were fasted for 6 hours prior to being injected at time 0 with 2 g/kg body weight of D-glucose (individually dosed). Blood glucose values were recorded at 15 min, 30 min, 60 min, 90 min, and 120 min post-injection. Blood glucose curves (A) and area under the curve (B) are means ± SEM from 5-6 mice per group. *, P<0.05 versus LF (-/+) CrPic. **, P<0.01 versus LF (-/+) CrPic. ***, P<0.001 versus LF (-/+) CrPic. #, P<0.05 versus HF (-) CrPic.

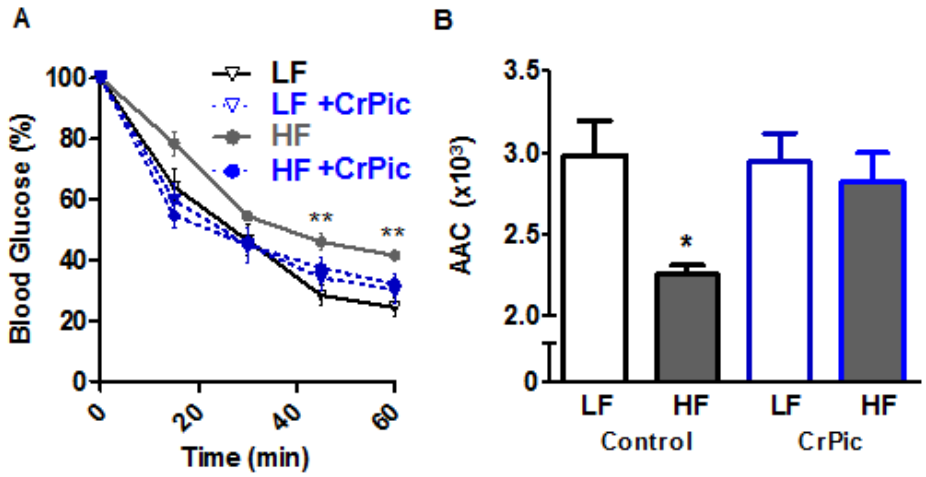

Figure 30. HF feeding promotes an impairment and CrPic supplementation improves glucose tolerance in C57Bl/6J mice. Intraperitoneal insulin tolerance tests were performed on mice following 8 weeks of dietary intervention. Mice were fasted for 6 hours prior to being injected at time 0 with 1 U/kg body weight of insulin (individually dosed). Blood glucose values were recorded at 15 min, 30 min, 45 min, and 60 min post-injection and reported as % change from basal. Blood glucose curves (A) and area above the curve (B) are means ± SEM from 4-6 mice per group. *, P<0.05 versus LF. **, P<0.01 versus LF.

hyperinsulinemia. Plasma insulin was found to be elevated in mice fed the HF diet, whereas this effect was not observed in animals fed a HF diet supplemented with CrPic (**Fig. 31**). Together, this data suggested that CrPic supplementation could improve glucose homeostasis.

HF Feeding Promotes and CrPic Supplementation Prevents against Increased HBP Activity and Cholesterol Accrual: Given the previous *in vitro* findings, study next sought to determine if CrPic supplementation *in vivo* could prevent against the progression of insulin resistance through inhibition of cholesterol accrual. To test this, upon sacrifice, epididymal fat was collected for analyses. Adipose tissue weight was significantly increased in mice fed a HF diet, yet CrPic supplementation did not any have an effect on this parameter. However, CrPic supplementation did protect against gains in adipose tissue membrane cholesterol accrual (**Fig. 32A**). To dissect if this increase could be due to a transcriptional response similar to that observed in adipocytes, mRNA levels of HMGR were assessed. HF feeding resulted in almost a 3 fold increase in the mRNA levels of HMGR, whereas CrPic supplementation prevented against this gain (**Fig. 32B**). In addition, phosphorylation of AMPK was found to be elevated in adipose tissue of animals fed a HF diet supplemented with CrPic (**Fig. 32C**). Consistent with the possibility of CrPic inhibiting the HBP through activation of AMPK, immunoprecipitation experiments carried out on adipose tissue revealed a significant increase in *O*-GlcNAc of Sp1 in HF mice that was prevented with CrPic supplementation (**Fig. 32D**). Together, these data suggest that CrPic may be exerting beneficial effects on glucose metabolism through

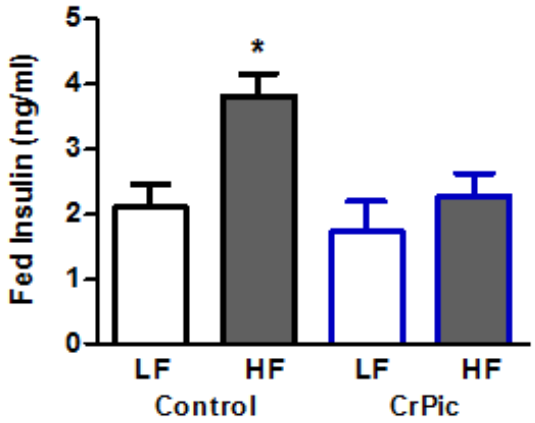

Figure 31. HF feeding promotes hyperinsulinemia. Random fed plasma was collected from mice following 8 of dietary intervention. An ELISA kit was used to measure the plasma insulin concentrations, which were determined by interpolation using a standard curve. Means values ± SEM are shown from 8-9 independent experiments. *, P<0.05 LF.

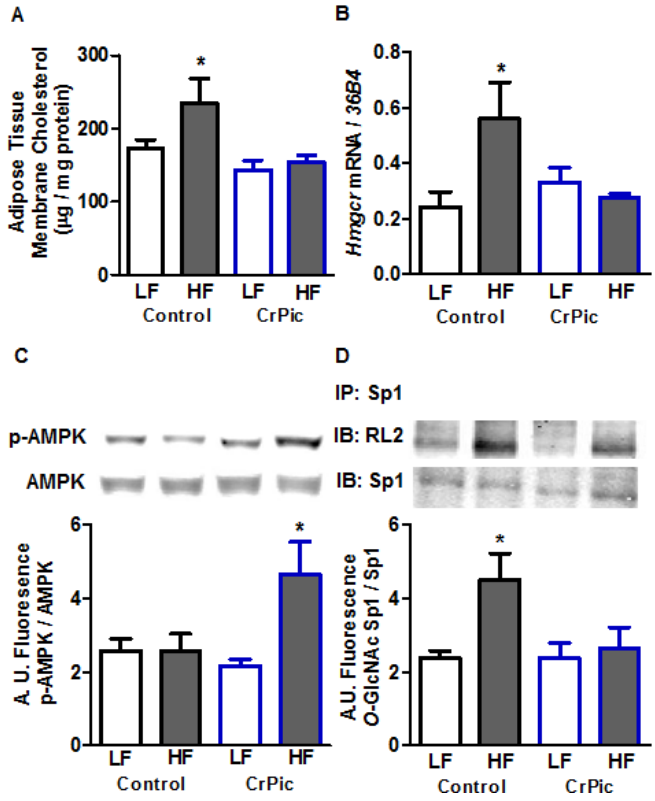

Figure 32. CrPic ameliorates increased HBP activity in HF fed mice. Epididymal fat pads were collected from mice in the post-prandial state after 8 weeks of dietary intervention. The tissue was homogenized and crude membrane fractions, purified RNA and whole cell lysates were prepared. Crude membrane cholesterol (A) was assessed and normalized to total membrane protein. Mean values ± SEM are shown from 6-12 mice. *, P<0.05 versus LF. The mRNA content of *Hmgcr* (B) was also determined and normalized to *36B4*. Means values ± SEM are shown from 7-9 mice. *, P<0.05 versus control. Protein levels of p-AMPK (C) were determined by immunoblotting, normalized to total AMPK. Mean values ± SEM are shown from 6 mice. *, P<0.05 versus control. Lysates were immunoprecipitated for detection of *O*-GlcNAc on Sp1 (D). Means values ± SEM are shown from 5-6 mice. *, P<0.05 versus control.

attenuating glucose flux through the HBP, thereby inhibiting ectopic membrane cholesterol accumulation and impaired glucose transport.

In summary, data from these *in vitro* and *in vivo* studies presented in Chapter IIC offer several novel observations. They first postulate a potential mechanism whereby chromium may mitigate an increased transcriptional response leading to the development of insulin resistance. The evidence presented here suggests this could be mediated through an inhibition of the HBP, either globally or through specific inhibition of the *O*-GlcNAc status of Sp1. Additionally, study conducted in this chapter suggests that high fat feeding *in vivo*, known to activate the HBP (356), may also promote insulin resistance through activation of cholesterolgenic machinery. Chromium supplementation in these mice resulted in a protection against the exacerbation of insulin resistance. In particular, CrPic was shown to activate AMPK in mice fed a HF diet. Potentially, this could inhibit HBP activity via phosphorylation and inactivation of GFAT (**Fig. 33**). Regardless of the precise mechanism, CrPic supplementation reduced the transcriptional activation of HMGR mRNA induced by HF feeding. This was also associated with a loss in the activity of the HBP, suggesting a new mechanism for the protective action of CrPic *in vivo*.

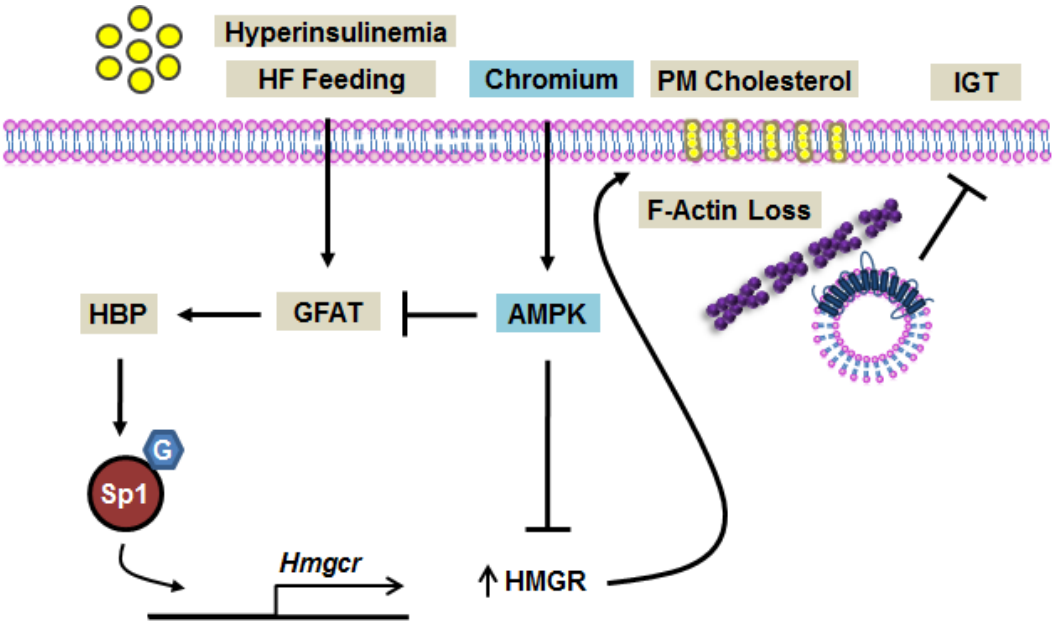

Figure 33. Model of hyperinsulinemia or HF feeding induced insulin resistance and protective actions of CrPic. Exposure of 3T3-L1 adipocytes to hyperinsulinemia induces increased HBP activity and pronounced activation of cholesterolgenic transcription factors such as HMGR. This results in a buildup of PM cholesterol which impairs distal regulation of GLUT4 translocation and glucose uptake. CrPic inhibits HBP activity, preventing the increased mRNA and protein levels of HMGR as well as PM cholesterol accrual. CrPic corrects glucose transport dysfunction induced by hyperinsulinemia independently of changes in insulin signaling. *In vivo* analyses of mice fed a HF diet suggest a potential role of this pathway in promoting cholesterol synthesis and accrual. CrPic supplementation in mice protects against increased HBP activity, cholesterol accumulation and the development of insulin resistance. The mechanism of action of CrPic may entail its known activation of AMPK both *in vitro* and *in vivo*, thereby potentiating flux through this pathway.

Chapter III. Perspectives

The findings presented above in Chapter II have provided insight into the mechanisms by which hyperinsulinemia promotes cholesterol synthesis through engaging the HBP. This increased deposition of cholesterol onto the PM promoted an impairment in glucose transport and increased endosomal membrane cholesterol impaired the ability of adipocytes to efflux cholesterol, perhaps exacerbating this cycle. In addition, they provided evidence that this process also occurs to some degree *in vivo*, and that CrPic supplementation impinges on the HBP and this may explain its mechanism of action on improving glucose transport. The following section will consider future research questions that stem from the current research. It will also serve to place these current findings into perspective given the numerous studies in the field discussed in the introductory sections. As previously described, the mechanisms by which the intake of excessive nutrients and the composition of these nutrients can contribute to glucose intolerance are complex and highly interdependent. Findings from the present studies suggest a key role for the HBP in promoting many of the phenotypical features associated with insulin resistance. As previously highlighted, the importance of distal mechanisms involved in insulin signaling for GLUT4 translocation has been a subject of recent research (reviewed in (80, 356)). Emerging interest in understanding these mechanisms has been fueled by the potential to provide novel downstream therapeutic targets that could potentiate insulin action. In this regard, study in our lab has demonstrated that pathophysiologic hyperinsulinemia, thought to be an early or perhaps primary derangement in the etiology of disease, promotes insulin

resistance despite intact signaling processes (8, 12). Rather, our data suggest that hyperinsulinemia may promote insulin resistance through altering PM architecture necessary for distal components of insulin regulation of GLUT4 exocytosis.

To first establish the relationship between hyperinsulinemia and alterations in PM cholesterol content in Chapter IIA, study sought to demonstrate the role of low pathophysiologically relevant doses in promoting a cholesterolgenic response. The doses utilized in this study were consistent with other reports that have shown that low doses of insulin are sufficient to induce insulin resistance (8, 12, 13, 151). Of note, while a 250 pM dose of insulin was found to result in a submaximal gain in PM cholesterol content, it did not have any observable effect on generation of HMGR. This was consistent with its inability to promote increases in SREBP1 content. While we did not test the effects of this dose on glucose uptake, we expect that it may have very modest, if any effects on this parameter based on the transcriptional findings. While this dose was in the upper quartile range of what the San Antonio Heart study of approximately 3000 participants defined as hyperinsulinemia (135), it also fell within the range of fasting insulin levels (6-300 pM), suggesting this dose is too low to promote the derangements observed in our model system. Another consideration is that the 3T3-L1 adipocytes have been previously reported to contain detectable levels of intracellular insulin degrading enzyme activity which could contribute to a gradual decline in insulin levels over time (357). Nevertheless, the observed effects with concentrations of insulin akin to those *in vivo* suggest that degradation rates were likely negligible in this system.

Studies showing that hyperinsulinemia can promote increases in HBP activity, provoking *O*-GlcNAc modification of Sp1, are consistent with previous reports that this transcription factor is modified at several residues by OGT (332). However, while other groups have demonstrated this modification of Sp1 in other cell types serves to translocate Sp1 to the nucleus (358, 359), this observation was not detected in current study. Yet other study has demonstrated that Sp1 is primarily localized to the nucleus (333), which could make any subtle changes difficult to detect. Nevertheless, SREBP1, known to be regulated by Sp1, was found to be elevated in the nuclear fractions upon treatment with hyperinsulinemia. Although the nuclear status of SREBP2 was also examined, as it plays a greater role in cholesterol synthesis, no alterations were observed in total or nuclear content. One report conducted *in vitro* binding assays with *O*-GlcNAcylated Sp1 and found that the modified form of Sp1 failed to bind to SREBP2 (360). Whether or not this binding is necessary for a maximal activation of the promoter region of SREBP2 is currently unknown. The increases in SREBP1 content in the nucleus are hypothesized to be the consequence of either the increased binding of Sp1 to the promoter region of SREBP1, known to activate it, or perhaps due to insulin's ability to regulate the degradation of Insig (182, 183). Studies with DON demonstrate a reduction in PM cholesterol content, favoring the former hypothesis as DON was shown to reduce the affinity of Sp1 toward the promoter of SREBP1. Alternatively, these perturbations in the regulation of SREBP1 could also promote saturation of Insig, thereby increasing SREBP1 processing (183).

Since the discovery that Sp1 was a target of OGT, leading to increases in its stability, several reports have hypothesized that the modification of this protein might induce alterations in its ability to bind to DNA as well as promoter activity (361-363). In fact, clinical findings underscore this importance as mutations in the Sp1 binding sites have been observed to correlate with a significant risk for the development of diabetic complications such as nephropathy (364). In addition, sequencing analyses have revealed a novel β cell glucokinase mutation in the promoter region of this gene (365). In this study, the mutation was mapped to a binding site of Sp1, and the promoter activity in patients with this mutation was reduced up to 4 fold. This resulted in a shift in the set point for insulin secretion to 7 mM, promoting fasting hyperglycemia. In the context of provoking cholesterol synthesis, ChIP analyses demonstrated that hyperinsulinemia-induced elevations in *O*-GlcNAc on Sp1 translated to its increased binding affinity to the promoters of both SREBP1 and HMGR. Previous studies have demonstrated that SREBP1 contains 5 Sp1 binding sites which, in conjunction with SREBP1, LXR, and NF-Y, drive its expression (209-212). The increases in SREBP1 in the nucleus, perhaps caused by Sp1 mediated activation of SREBP1, could then potentially result in its collaboration with Sp1 bound to the HMGR promoter to further increase the synthesis of HMGR as well. In addition, study has demonstrated that LXR is a target of OGT, and this modification leads to increased expression of SREBP1 (215). Together, these results lend further support that *O*-GlcNAc could play a central role in activating a cholesterolgenic response through the modulation of these critical transcription factors implicated in increased cholesterol synthesis.

A potential caveat to the study of precisely how *O*-GlcNAc modification of Sp1 affects its function is that the RL2 antibody and Click-iT kits employed in Chapter IIA studies do not differentiate between the various sites that are modified by *O*-GlcNAc. To date, at least 8 residues on Sp1 have been identified as targets for this modification, with a majority of them being in the DNA binding domain (366). Five of the sites for *O*-GlcNAc, in fact, have been identified to be in the zinc finger DNA binding domain of Sp1, and mutating these sites to alanine residues resulted in reductions in basal transcriptional activity (330). In contrast, mutagenesis of a peptide derived from the Sp1 activation domain also suggests that *O*-GlcNAc modification in this region may inhibit protein-protein interactions necessary for transcriptional activation (205, 360, 367, 368). These findings thus suggest a need in the field to identify precisely which residues are modified in the presence of hyperinsulinemia. Yet the mapping of these modified proteins remains limited at a technological level by the fact that *O*-GlcNAc is unstable during collision-induced dissociation in mass spectroscopy (369-371). Recently, new methods have been implemented to assist in this process, in particular chemical/enzymatic photochemical cleavage techniques that introduce a photochemical cleavable biotin probe on *O*-GlcNAc residues (372-375). This method and other similar enzymatic labeling methods are estimated to have a sensitivity in the low femtomole range, overcoming the limitation of needed milligram quantities of protein to detect sites of glycosylation. Future studies are thus warranted that will examine precisely what residues are modified by *O*-GlcNAc and how they affect protein-protein interactions and DNA binding in the context of hyperinsulinemia-induced insulin resistance.

The observation in Chapter IIA that HMGR protein levels are elevated by hyperinsulinemia treatment and corrected by HBP inhibition is also somewhat perplexing given the intense regulatory mechanisms discussed in the introduction to maintain precise cholesterol homeostasis. An initial hypothesis was that perhaps cholesterol levels were increased solely at the PM such that endosomal cholesterol content was normal, preventing the degradation of HMGR known to occur when endosomal cholesterol levels rise. Yet studies presented in Chapter IIB refute this hypothesis and rather suggest global increases in cellular cholesterol. Future research endeavors could seek to assess the rate of turnover of HMGR, while also examining the status of other proteins involved in cholesterol synthesis. A hypothesis favored in this regard is that HMGR turnover is likely increased in cells treated with hyperinsulinemia, but perhaps the increased rate of synthesis overcomes the degradation. Another potential explanation for the increase in cholesterol is through the process of macrolipophagy in which cholesterol esters and triglycerides stored in lipid droplets become mobilized (376). Indeed, study from Timothy Osborne's lab suggests that this process can contribute to increases in cellular cholesterol content (377). Yet these studies suggested that this process is mediated by SREBP2 and occurs in situations of sterol depletion. Nevertheless, whether SREBP1 could play a role in this process and if the HBP could provoke this aberrant response in adipocytes despite adequate cholesterol content is a subject of research. Importantly, inhibition of the HBP with DON attenuated cholesterol synthesis at the level of transcription, providing further evidence that

the insulin-induced defects are likely functioning at the transcriptional level to promote gains in HMGR and PM cholesterol content.

Data presented in Chapter IIA also suggest a central role of Sp1 in triggering the cholesterolgenic response as indicated by the experiments conducted using mithramycin. Study has previously demonstrated a major role of Sp1 in regulation of resistin expression (331, 378). In this regard, mithramycin has also been shown to inhibit the transcription and secretion of interleukin 6 (IL-6), a pleiotropic cytokine known to impair glucose-stimulated insulin secretion in rodents (379-381), and insulin action in adipocytes (382-384). In the current studies, mithramycin abrogated the hyperinsulinemia-induced increases in HMGR protein levels, restored PM cholesterol and F-actin structure, and improved glucose transport rendered impaired by hyperinsulinemia. The observed effects of mithramycin were not attributable to a loss of *O*-GlcNAc on Sp1 as this was found to be elevated despite mithramycin treatment. This data suggested a potential critical role of Sp1 in the dysregulation of cholesterol synthesis that occurs with altered nutrient delivery to adipocytes.

Together, the data presented in Chapter IIA suggest that Sp1 may be a potential therapeutic target for improving insulin sensitivity. Other study with mithramycin has shown that it may have beneficial effects on hepatic insulin signaling through its negative regulation of protein phosphatase 1B (385). As previously mentioned, Sp1 binding sites have also been found on a host of promoters encoding adipokines including leptin, resistin, and adiponectin (331, 363, 364, 378). Of particular interest is the finding by one study that suggests PPARγ agonists specifically inhibit *O*-GlcNAc modification of Sp1 (331), thereby

exerting a positive effect on insulin sensitivity in adipocytes. In this regard, we hypothesized that HBP inhibition could protect against insulin resistance through inhibiting Sp1 modification and that increased HBP might be responsible for impairments in cholesterol efflux processes in addition to impaired glucose transport.

The studies presented in Chapter IIB were performed in direct collaboration with a fellow post-doc in the lab, Whitney Sealls. In Chapter IIB, the role of cholesterol accrual in mitigating cholesterol efflux processes in adipocytes was further examined. In addition, the role of CrPic treatment in alleviating these cellular derangements and its potential mechanism were explored. The basis for these CrPic studies comes from early studies in humans suggesting that CrPic deficiency is linked to reductions in circulating HDL (386). Additionally, brewer's yeast in human trials has been shown to improve plasma HDL concentrations (220, 221). While still fragmentary, even Nanne Kleefstra, who has suggested in several reports that human CrPic trials should conclude no effect on any parameter of glucose metabolism, observed a trend toward increased HDL in patients receiving CrPic intervention (1.15 ± 0.26 mmol/l in placebo and 1.47 ± 0.54 mmol/l in the intervention group), lending support for a potential beneficial role in humans (387). Regardless, an emerging appreciation is that total HDL measurements are misleading in understanding its cardioprotective actions, as the ABCA1-generated pre-β-1 HDL particle likely represents the "functional" subfraction (339).

Mechanistically, it was determined that CrPic supplementation improves cholesterol efflux processes in adipocytes, perhaps directly through lowering

endosomal cholesterol content and thereby allowing for appropriate extraction of Rab8 by GDI. Support for the role of endosomal cholesterol accrual in this process comes from the fact that CrPic was associated with a correction of endosomal membrane cholesterol levels and that HBP inhibition also improved cholesterol efflux. Additionally, numerous studies have demonstrated that endosomal motility is impaired in NPC as well as cells treated with U18666A, a cholesterol transport inhibitor (388-390). In these studies, it was suggested that the impairment was due to an inhibition of the membrane-cytosol cycling of Rab7, resulting in reduced dynamics. Moreover, other study has further supported this hypothesis through the examination of viral infectivity. In one such study, the Vesicular Stomatitis Virus (VSV) was utilized, which infects cells from the endosomes (391). In these studies, it was found that the action of this virus was significantly attenuated in U18666A treated cells, suggesting that cholesterol accumulation impairs membrane dynamics. Yet this treatment did not affect the fusion of the virus with the endosomes, but rather VSV infectivity, presumably because the capsids released into the endosomal membrane remained trapped due to impaired trafficking processes. As such, future studies with regard to cholesterol efflux could be performed to specifically determine if increases in endosomal cholesterol content impair the cycling of Rab8. Of particular interest is whether extraction of Rab8 by GDI may become impaired, as others have shown occurs with Rab9 during cholesterol accumulation observed in NPC (341).

Of clinical importance, the development of pro-atherogenic foam cells is thought to result from the impaired ability of macrophages to efflux cholesterol via ABCA1 and ABCG1 (392). If this efflux of cholesterol is sufficient to rid oxidized

cholesterol generated from reactive oxygen species in the neointima, the development of atherosclerosis can be circumvented. In this context, a loss in the generation of pre-β-1 HDL by adipocytes could contribute to the progression of atherosclerosis and the development of coronary artery disease (CAD). A potential caveat to translating this finding to humans is data by Ishida that suggests that total pre-β cholesterol makes up as much as 63% of plasma ApoA-I in lipoprotein disorders such as familial hypercholesterolemia, lipoprotein lipase deficiency, LCAT deficiency, and familial combined hyperlipidemia (393). In addition, since the discovery of methods to segregate and resolve pre-β-1 HDL through nondenaturing electrophoresis, a paradoxical connection between elevated levels of pre-β-1 HDL, CAD, and increased carotid intima-media thickness has been observed (394). Several other studies have reported this elevation in patients with coronary heart disease (395, 396), and a recent clinical trial involving 1255 subjects suggests this fraction as an independent risk factor for this disease as well as myocardial infarction (397). Importantly, however, this accumulation itself is postulated to be compensatory rather than causative in this regard as study suggests that the accumulation may result from a defect in LCAT activity seen in patients with CAD (398). Interestingly, exercise, known to stimulate AMPK activity, has been shown in type 2 diabetics to enhance the conversion of pre-β-1 HDL into its more mature forms, thereby potentially leading to an improvement in cardiovascular outcomes (399). These findings suggest future studies with chromium to determine if its activation of AMPK may not only improve the generation of pre-β-1 HDL for macrophage efflux but also increase

its conversion by LCAT, thereby potentially improving neointimal thickness, macrophage efflux, and CAD.

Of importance for future study is the potential that impaired ABCA1-mediated cholesterol efflux may potentiate the pathogenesis of diabetes through further accumulation of cellular cholesterol content. Additionally, defects in ABCA1-mediated cholesterol efflux in β cells are known to contribute to impaired insulin secretion (222). In fact, a small study of patients with Tangier's disease, caused by a mutation leading to a defective ABCA1 transporter, revealed impaired glucose tolerance and insulin secretion (400). An important direction of future research will be to generate a unique ABCA1 inducible knockout mouse specific to adipose tissue or skeletal muscle. This will corroborate current cell culture analyses presented in Chapter IIA and B which suggest elevations in cholesterol content may impair glucose uptake and cholesterol efflux processes.

In Chapter IIC, the mechanism(s) of action of CrPic on benefiting glucose metabolism was examined *in vitro* and *in vivo*. These studies suggested a novel mechanism whereby CrPic my impart protection against glucose intolerance through modulation of cholesterol synthesis. Further, data presented in this section sought to test whether CrPic inhibits glucose flux through the HBP, thereby decreasing *O*-GlcNAc of Sp1 and inhibiting transcriptional activation of cholesterol synthesis. In line with cell culture studies, animal data presented support the possibility that, in part, cholesterol accumulation *in vivo* may arise from an increase in cholesterol synthesis provoked by elevations in HBP activity.

An important finding in these studies was that neither CrPic nor hyperinsulinemia had any effect on distal components of insulin signaling. While

proximal insulin signaling was not examined in the current study, previous reports have shown that neither CrPic nor hyperinsulinemia alone promoted amplification or impairment, respectively, in these proteins (8, 12, 21, 22), making it unlikely that a combinatorial effect would be observed proximally that would not contribute to a distal defect. These findings are also consistent with the idea that distal components of GLUT4 regulation at or near the PM are important targets for impairments in insulin action (80).

The findings in Chapter IIC implicate that CrPic may impinge upon the HBP through its activation of AMPK, observed in both HF fed animals supplemented with CrPic and in cell culture systems. While recent reports suggest that AMPK may phosphorylate GFAT and inhibit HBP activity in this manner (309, 310), this process was not examined in the current study. Of interest, however, were parallel studies conducted in L6 myotubes which highlight a requirement for AMPK in this micronutrient's mechanism of action. In these studies, myotubes treated with CrPic displayed elevated AMPK activation, as evidenced by increased phosphorylation of the catalytic alpha subunit at threonine 172. The effect of CrPic-induced phosphorylation of AMPK was demonstrated to be more robust in cells that were cultured in hyperinsulinemic conditions. To determine if AMPK activity was required for the beneficial aspects of CrPic action on glucose metabolism, siRNA technology was employed to knock down the catalytic α subunit of AMPK. This knockdown experiment resulted in a reduction in the detection of AMPK protein by approximately 80% (**Fig. 34**). Consistent with AMPK mediating the action of CrPic, in cells transfected with scramble oligonucleotides, CrPic treatment was able to restore a loss in GLUT4

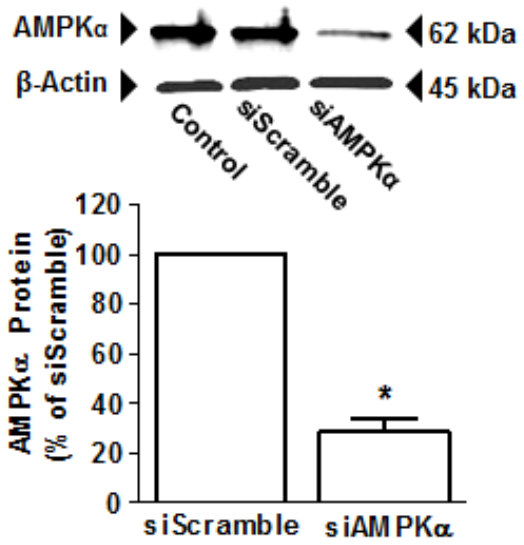

Figure 34. AMPKα knockdown efficiency. Following transient transfection with control oligonucleotides (siScramble) or those targeting the α catalytic subunit of AMPK (siAMPK), whole cell lysates were prepared and subjected to immunoblotting to assess total protein content of AMPK as a measure of efficiency of the knockdown. Means values ± SEM are shown from 4 independent experiments.

redistribution to the PM induced by hyperinsulinemia (**Fig. 35A**). Contrary to this, cells that were treated with siAMPK had a marked reduction in the ability of insulin to stimulate GLUT4 translocation that was not corrected by treatment with CrPic (**Fig. 35B**). To corroborate these findings and investigate the functionality of GLUT4, glucose uptake assays were performed. As observed with the GLUT4 translocation assays, an impairment was seen in the responsiveness of insulin to stimulate glucose uptake in the presence of hyperinsulinemia (**Fig. 35C**). This was corrected with CrPic treatment in these control transfected cells, but not in the cells in which AMPK was knocked down with siAMPK (**Fig. 35D**). Importantly, insulin signaling remained intact in these knockdown cells, and CrPic did not result in the phosphorylation of residual AMPK. This data thus strongly supports a causative role of AMPK in mediating the beneficial aspects of CrPic on glucose metabolism. This is in line with a recent study demonstrating that inhibition of AMPK with compound C blunts the effect of CrPic inhibition of resistin secretion in adipocytes (281). Future studies should therefore determine if this beneficial aspect of CrPic involves the inactivation of GFAT.

Our studies using HF fed mice to induce the development of insulin resistance lends further support to the idea that CrPic could counter against elevated HBP activity induced by HF feeding. Indeed, in transgenic mice overexpressing GFAT, HF feeding is not additive in causing insulin resistance, consistent with a shared pathway (401). In addition, GFAT overexpression specifically in the adipose tissue of mice results in increased serum leptin, decreased adiponectin, increased fat synthesis, as well as skeletal muscle insulin resistance as assessed by both hyperinsulinemic-euglycemic clamp as well as

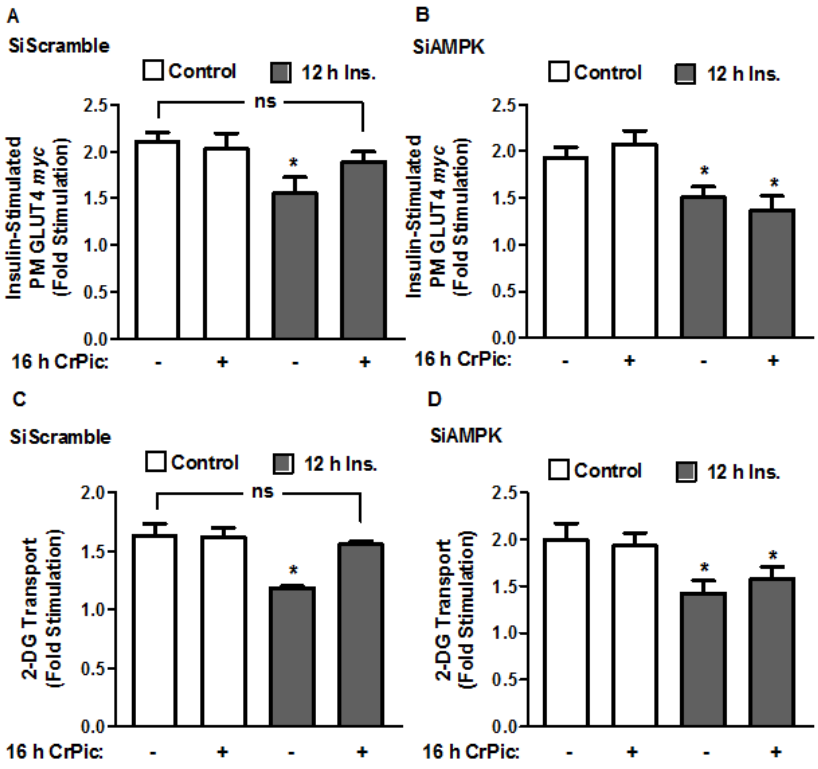

Figure 35. AMPKα is necessary for the protective actions of CrPic on insulin responsiveness. Following transient transfection with control oligonucleotides (siScramble) or those targeting the α catalytic subunit of AMPK (siAMPK), GLUT4 *myc* and 2-DG transport assays were performed. L6 myotubes were pretreated with or without 10 nM CrPic for 16 h. After 4 h of pretreatment, cells were treated with or without 5 nM insulin for 12 h. Following treatments, cells were placed in serum free media for 1 h. During the last 20 min, cells were incubated with 100 nM insulin to stimulate GLUT4 translocation and glucose uptake. The fold increase in insulin-stimulated GLUT4 *myc* immunofluorescence was determined in siScramble (A) and siAMPK (B) as well as 2-DG transport in siScramble (C) and siAMPK (D) cells. Means values ± SEM are shown from 6-8 independent experiments. *, P<0.05 versus insulin-stimulated control.

2-DG uptake from hind limb muscle (156, 158). These studies, coupled to thoseinducing overexpression of GLUT1 (402), suggest a prominent role of elevations in HBP pathway in adipose tissue in promoting derangements in whole body glucose disposal.

In terms of human health, these data presented in Chapter IIC suggest a novel, putative mechanism by which CrPic could be beneficial to glucose metabolism via countering PM stress. While clinical trials suggest a beneficial effect of CrPic in diabetics, it may be possible that CrPic supplementation has an effect to counter dysregulation in membrane fluidity and that this incremental affect may become lost later in disease progression when other factors further dampen insulin sensitivity. Alternatively, other drugs patients are taking, such as metformin, known to activate AMPK, may mask the effects of CrPic action. Further, well-designed longitudinal studies are thus needed in insulin resistant, non-diabetic patients to help better characterize the role of this micronutrient in alleviating insulin resistance.

Conclusion

In conclusion, together the data presented in Chapter II provide novel mechanistic insight into how hyperinsulinemia may promote cholesterol accumulation associated with impaired glucose and lipoprotein metabolism. In this context, the data has indicated an important role for alterations in HBP activity in mediating these consequences. In addition, these data demonstrate that CrPic treatment can alleviate these alterations in cholesterol efflux and glucose transport, as well as improve glucose tolerance and insulin sensitivity in

a diet-induced mouse model of insulin resistance. Data presented in Chapter IIA mechanistically link elevations in HBP activity in promoting a transcriptional response leading to increases in cholesterol synthesis. Inhibition of this pathway or direct inhibition of a direct target of this pathway, Sp1, prevented against this response and restored insulin responsiveness in adipocytes. Expanding on these findings, studies presented in Chapter IIB suggest that HBP-induced cholesterol accrual may impair the appropriate trafficking of ABCA1, perhaps due to an alteration in the proper extraction of Rab8. These studies also highlighted a beneficial role of CrPic treatment or HBP inhibition in decreasing endosomal cholesterol content, strengthening the hypothesis that CrPic may inhibit cholesterol synthesis through altering flux through the HBP. In this manner, CrPic also rescued an impairment in cholesterol efflux induced by hyperinsulinemia. In Chapter IIC, it was found that CrPic appears to inhibit flux through this pathway both *in vitro* and *in vivo*. This was associated with a loss of transcriptional activation of cholesterol synthesis, restoration of PM cholesterol, and improvement in glucose uptake. CrPic supplementation in mice was found to blunt the development of insulin resistance induced by HF feeding, perhaps mediated in part through the same mechanism. These data suggest that CrPic, through AMPK activation, suppresses cholesterol synthesis through inhibition of the HBP, thereby lowering membrane cholesterol that disrupts F-actin structure and induces insulin resistance in adipocytes and adipose tissue.

Chapter IV. Experimental Procedures

Cell Culture and treatments

Murine 3T3-L1 preadipocytes were purchased from Dr. Howard Green (Harvard Medical School) and cultured as previously described (403). Briefly, 3T3-L1 preadipocytes were cultured in DMEM containing 25 mM glucose and 10% calf serum in at 37°C at a 10% CO_2 atmosphere. Confluent cultures were induced to differentiate into adipocytes by incubation of the cells with DMEM containing 25 mM glucose, 10% fetal bovine serum, 1 µg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-1 methylxanthine. After 4 days, the medium was changed to DMEM containing 25 mM glucose, 10% fetal bovine serum, and 1 µg/ml insulin for an additional 4 days. All studies were performed on adipocytes between 8 and 12 days post initiation of differentiation. The cells were left untreated or treated for 12 h with insulin (250 pM - 5000 pM) in serum free DMEM medium. Overnight insulin incubations were limited to 12 h to minimize complications on glucose transport due to glucose deprivation (404). Several pretreatment conditions were also tested. These pretreatments were for 16 h and included 20 µM DON, 100 nM mithramycin, and 10 nM CrPic. Additionally, study also utilized a 12 h treatment with 300 µM methyl-β-cyclodextrin. Studies utilizing AICAR were conducted by incubating cells in the presence of AICAR for the last 45 min of the study. Acute insulin stimulation was performed by treating adipocytes with a 100 nM dose of insulin during the last 5 min for insulin signaling or 30 min for glucose transport analyses.

For studies in L6 myotubes, rat L6 muscle cells stably expressing GLUT4 that carries an exofacial *myc*-epitope (provided by Dr. Amira Klip) were cultured as previously described (11). Briefly, myoblasts were cultured in an α-minimum essential medium (α-MEM) containing 5.5 mM glucose and 10% fetal bovine serum. Cells were differentiated into multinucleated myotubes by placing them in α-MEM containing 5.5 mM glucose and 2% fetal bovine serum. All studies were performed on myotubes between 4 and 6 days post-initiation of differentiation. The cells were left untreated or treated for 12 h with insulin (5 nM) in DMEM containing 5 mM glucose and 1% fetal bovine serum. Pretreatment included the incubation of myotubes for 16 h with 100 nM CrPic. Acute insulin stimulation was performed by treating myotubes with a 100 nM dose of insulin during the last 20 minutes for glucose transport analyses.

Mice, diets and CrPic supplementation

Male C57Bl/6J mice (age 4 weeks) were obtained (Jackson Laboratory, Bay Harbor, Maine) and were singly housed in a light and temperature-controlled animal facility (12/12 (light/dark), 20°C). All mice had free access to water with or without CrPic that provided approximately 8 µg/kg body weight per day. Based on the fact that commercially available nutritional supplements generally contain CrPic at 200 to 600 µg/kg per day and an average human mass of 75 kg, the supplements provide 600 µg/kg per day. All animals received standard laboratory chow for 2 weeks followed by a LF diet containing 20% kcal from protein, 70% kcal from carbohydrates, and 10% kcal from fat (D01030107, Research Diets Inc., New Brunswick, NJ). This LF, as well as the HF diet represented modified

forms of the standard LF (D12450B) and HF (D12451) diets with adaptions regarding type of fat (palm oil instead of lard) and carbohydrates to better mimic the FA and carbohydrate composition of the average human diet in Western societies. The mice were fed the modified LF diet for 3 weeks to adapt. After this 5 week total acclimation period, mice were either left on the LF diet or switched to the HF diet containing 20% kcal from protein, 35% kcal from carbohydrates, and 45% kcal from fat (D01030108). By addition of chromium sulfate to the chromium-free diets, both the LF and HF test diets were controlled to contain an equal amount of chromium (1.9 mg/kg diet). Note that the energy density of all nutrients, except fat and starch, is equal. All animal protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

IPGTT and IPITT

C57Bl/6J mice were fasted for 6 hours (from 8:00 a.m. to 2:00 p.m.). Mice were then injected intraperitoneally with 2 g/kg D-glucose (individually-dosed) at time 0 for the IPGTT and blood was obtained vial tail collection at 15, 30, 60, 90, and 120 minutes post-injection of glucose. Mice were injected with 1.0 unit of insulin/kg (individually-dosed) at time 0 for IPITT, and blood was obtained via tail collection at 15, 30, 45 and 60 minutes post-injection of insulin. Blood glucose was analyzed using the Abbott Animal Health AlphaTRAK blood glucose meter (Abbott Laboratories, Abbott Park, IL).

Insulin ELISA

Upon sacrifice, blood was collected from mice by cardiac puncture in the fed state. Plasma was obtained by centrifuging blood samples at 4°C for 15 minutes at 2000 revolutions per minute using a table top centrifuge. Plasma insulin concentrations were determined using the low range assay (0.1-6.4 ng/ml) protocol from the Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem. Inc., Downers Grove, IL).

Subcellular fractionation

Purified adipocyte endosomal membrane, cytosol, and plasma membrane fractions and epididymal adipose crude membrane fractions were prepared as described previously (405). Purified fractions were resuspended in a detergent containing lysis buffer and recovered protein content was determined using the Bradford method. Nuclear extracts were collected as previously described (406). Briefly, 3T3-L1 adipocytes were washed with PBS and collected in 2.0 ml of hypotonic buffer (10 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1mM DTT containing 10 mM leupeptin, 2 mM pepstatin, 2 mM aprotinin, and 0.5 M PMSF). Lysates were prepared by passing the cells through a 22-gauge needle 5 times, followed by centrifugation at 800 g for 10 min at 4°C. The pellet was then resuspended in 200 µl of buffer C (10 mM HEPES, pH 7.4, 0.42 M NaCl, 25% glycerol (v/v), 1.5 mM $MgCl_2$, 0.5 mM EDTA containing 10 mM leupeptin, 2 mM pepstatin, 2 mM aprotinin, and 0.5 M PMSF). Proteins were extracted from the nuclei with gentle vortexing for 15 s every 10 min for 1 h. The mixture was then centrifuged at full speed in a microcentrifuge at 4°C and the supernatant

consisting of nuclear proteins was collected for Bradford method to determine protein content.

Actin analyses

Actin labeling of 3T3-L1 adipocytes was performed as previously described (7). Briefly, after treatments cells were fixed in a 4% paraformaldehyde 0.2% Triton X 100 (v/v) solution containing PBS. Following fixation, cells were incubated with 5 µg/ml of FITC-conjugated phalloidin for 2 h. All cell images were obtained using a Zeiss LSM 510 NLO confocal microscope (Carl Zeiss, Thornwood, NY), and all microscope settings were identical between treatment groups. Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain (Molecular Probes).

Cholesterol analyses

3T3-L1 adipocytes PM or adipose tissue crude membrane pellets obtained from differential centrifugation were resuspended in 0.2 ml of HES buffer, and cholesterol content was assayed using the Amplex Red Cholesterol Assay Kit (Molecular Probes), as previously described (7). Briefly, 0.15 ml of the resuspended PM pellet was vigorously mixed with 3.0 ml of chloroform-methanol (2:1 v/v) for 10 min to extract cholesterol. The mixture was then centrifuged at 580 x g for 10 min followed by collection of 1 ml of the lower phase which was evaporated at 100°C for 10 min. The residue was then reconstituted with 0.1 ml of an isopropranol-Triton X solution (10:1 v/v) and 0.05 ml of the sample was incubated with 0.05 ml of Amplex Red cholesterol reaction buffer at 37°C for 30

min. After incubation, the absorbance was measured at 600 nm. Cholesterol content was normalized to total PM or crude membrane protein.

Protein analyses

After collection of cellular or adipose tissue lysates, membrane, cytosolic, or nuclear fractions, 30-50 µg of protein were separated on a 7.5% SDS-polyacrylamide gel (6% SDS for ABCA1 analyses), and resolved fractions were transferred to nitrocellulose (Bio-Rad, Hercules, CA). Membranes were then probed utilizing antibodies for HMGR (Millipore), SREBP2 (Abcam), SREBP1 (Abcam), ABCA1 (Abcam), Rab8 (BD Biosciences), or AMPK (Cell Signaling) followed by an IRDye™ 700DX or 800DX conjugated secondary (Rockland). Immunoblots were quantitated using a LI-COR Odyssey infrared imaging system. Immunoblots were normalized to β-actin (Cytoskeleton) for equal protein loading. For immunoprecipitation experiments, lysates from 3T3-L1 adipocytes or adipose tissue were collected using an NP-40 lysis buffer (25 mM Tris, pH 7.4, 137 mM NaCl, 10 mM NaPP, 1% NP40, 1% glycerol, containing 10 mM leupeptin, 2 mM pepstatin, 2 mM aprotinin, and 0.5 M PMSF). Whole cell lysates were then prepared by centrifugation at 14,000 rpm at 4°C for 15 min. The Bradford assay was performed to ensure equal protein loading for IP analysis. Immunoprecipitation was carried out overnight with 1.25 mg protein and 2 µg of antibody for Sp1 (Santa Cruz). Samples were then incubated with 80 µl of protein G agarose (Roche) for 1 h with rotation at 4°C. Protein complexes were pelleted by centrifugation at 14,000 rpm at 4°C for 10 min, washed with NP-40 lysis buffer, and eluted with 200 µl LSB containing β-mercaptoethanol and 0.1 M DTT.

Samples were then run on a 7.5% SDS-polyacrylamide gel and immunoblotted with an RL2 antibody (Thermo Scientific). For Click-iT *O*-GlcNAC enzymatic labeling experiments, lysates were immunoprecipitated as described above. Prior to elution, samples were resuspended in 200 µl 1% SDS, 20 mM HEPES at pH 7.9 and labeled with biotin-alkyne using the Click-iT *O*-GlcNAc enzymatic labeling system and protein analysis detection kits (Invitrogen). The immunoprecipitated samples were then eluted as described above. Samples were then run on a polyacrylamide gel and analyzed using a streptavidin antibody (Abcam).

RNA analyses

3T3-L1 adipocytes were lysed using a Qiagen QIAshredder and RNA was isolated using an RNeasy mini kit (Qiagen). For adipose tissue analyses, RNA was collected using the RNeasy lipid tissue mini kit (Qiagen) Purified RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Reactions were performed in a 96-well plate using the ABI Prism 7000 Sequence Detection System (Applied Biosytems). Each reaction contained the following: 12.5 µl of SYBR GREEN (Applied Biosystems), 200 nM of each primer, 3 µl of cDNA, and RNase free water to a total volume of 25 µl. The PCR conditions used were 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 40 s. Cycle threshold values were obtained and normalized to *36B4*. The ΔΔCt method was used to determine relative expression levels. Primers utilized for these and ChIP analyses (below) are shown in Appendix A.

ChIP analyses

After treatments, 3T3-L1 adipocytes were fixed with 1% formaldehyde in PBS for 10 min. The reaction was then quenched with 50 mM glycine in PBS and subsequently washed two times with ice-cold PBS. Cells were then scraped in PBS plus protease inhibitor cocktail and centrifuged for 2 min at 2,000 rpm. The pellet was resuspended in 500 µl ChIP lysis buffer (50 mM Tris pH 8.1, 10 mM EDTA, 1% SDS plus protease inhibitor cocktail) and sonicated (10 pulses of 30 s on, 30 s off) and centrifuged at 14,000 rpm at 4°C for 10 min. Chromatin size was checked by running on a non-denaturing polyacrylamide gel to ensure that the average size was between 200-500 bp. Fragmented chromatin preparations (100 µl) were then diluted with ChIP dilution buffer (Millipore), and an input sample was collected. Samples were precleared with 60 µl of ChIP blocked protein G-Agarose beads (Millipore) for 1 h at 4°C with rotation prior to immunoprecipitation with antibodies to Sp1 (Santa Cruz) or IgG (Millipore) overnight. Samples were then incubated with 60 µl of protein G-Agarose beads for 1 h, then protein-DNA complexes collected by centrifugation. Immunoprecipitates were washed in low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and 2 washes in TE buffer (Millipore, 5 min each). Immune complexes were then eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$), and reverse cross-linked with the addition of 8 µl of 5 M NaCl at 65°C overnight. Successful immunoprecipitation was ensured by running samples on a polyacrylamide gel and probing with the Sp1 antibody. After RNase A and Proteinase K treatment, phenol/chloroform was used to purify DNA which was used as a template for qPCR under the following conditions: 15 min at 95°C

followed by 35 cycles of 15 s at 95°C, 30 s at 50°C, and 34 s at 60°C. The fold enrichment method was used to quantitate Ct values. Subsequent non-denaturing gel electrophoresis was performed on samples subjected to qPCR to confirm the product. Dissociation curves were also performed and an additional aliquot of DNA was subjected to nanodrop to ensure purity, 260/280 was 1.8-2.0.

Plasmids & luciferase reporter assay

For luciferase assays, the proximal promoter sequence (-284 to +36) of *Hmgcr* was PCR amplified from mouse genomic DNA. Promoter fragments were sequenced and cloned into pGL2B luciferase reporter plasmids (Promega, Madison, WI). Differentiated adipocytes were electroporated (0.16 kV and 960 µF) as previously described (7). Briefly, cells were trypsinized and pelleted by centrifugation at 1000 rpm. Pellets were resuspended in PBS and re-pelleted. Pellets were then resuspended in 1.0 ml PBS. For transfection, 50 µg of *Hmgcr* pGL_2B and 50 µg of phrl-minTK (Renilla) plasmid were used at a concentration of approximately 1 X 10^7 cells/0.5 ml. A single pulse was then applied using a Gene Pulser (Bio-Rad #1652076). The electroporated cells were then allowed to recover and plated into a 24 well plate. Experiments were started 16-18 h after electroporation by placing cells in the appropriate medium. After treatments, cells were lysed and assayed for promoter activity using the dual-luciferase reporter assay system (Promega, Madison, WI). Firefly luciferase activities were normalized to Renilla activity to control for differences in transfection efficiency.

GLUT4 analyses

In L6 myotubes, GLUT4 *myc* labeling was performed as previously described (12). Briefly, myotubes were fixed with 2% paraformaldehyde/PBS. After fixation, cells were then blocked for 1 h at room temperature in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE). Samples were incubated in a 1:50 dilution of primary antibody overnight at 4°C, followed by incubation at room temperature with infrared-conjugated secondary antibody for 1 h. Images were collected and quantified with the Odyssey system. Immunofluorescent intensity was normalized to intensity from Syto60, a fluorescent nucleic acid stain.

Glucose transport assay

Glucose uptake assays were performed as previously described (7). Briefly treated 3T3-L1 adipocytes were incubated in a KRPH buffer (136 mM NaCl, 20 mM HEPES, pH 7.4, 5 mM sodium phosphate, pH 7.4, 4.7 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$) for 15 min. Cells were then left untreated or stimulated with 100 nM insulin for 30 min and exposed to 50 µM 2-DG containing 0.5 µCi 2-[^{3}H] deoxyglucose (Perkin Elmer) in the absence or presence of 20 µM cytochalasin B. For L6 myotubes, treated cells were incubated in glucose-free buffer (125 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 2.6 mM $MgSO_4$, 25 mM HEPES, 2mM pyruvate, and 2% BSA) for 30 minutes, after which stimulation was carried out with 100 nM insulin for 20 min. Cells were then exposed to 2-DG containing 0.055 µCi/µl 2-[^{3}H] deoxyglucose and nonspecific uptake was quantitated via cell associated radioactivity in the presence of 20 µM cytochalasin B. For both cell types, after 10 min, uptake was terminated by aspiration and quenched with the addition of 1.0

ml of 10 µM cytochalasin B or 4 quick PBS washes for adipocytes and myotubes, respectively. Cells were then solubilized in 0.2 N NaOH or 1.0 N NaOH and [^{3}H] was measured by liquid scintillation. Counts were normalized to total cellular protein, determined by the Bradford method.

Cholesterol efflux

ApoA-I-mediated cholesterol efflux was determined as previously described (407). Briefly, adipocytes were labeled with 0.5 µCi/ml ^{3}H-cholesterol (Sigma Aldrich) for 24 hours in 25 mM glucose DMEM containing 0.2% BSA. Cells were then washed and treated as described. Cells were then incubated in 25 mM glucose DMEM containing 0.2% BSA and 10 µg/ml lipid-free ApoA-I for 4 hours. This was followed by measuring ^{3}H-cholesterol in the medium and in the cells. The percentage of acceptor-specific efflux was calculated using the following equation: medium/(medium+cells). Values obtained in the absence of acceptor were subtracted to account for non-specific ^{3}H-cholesterol efflux/leakage.

siRNA design and transfection

Three independent oligonuceotide sequences, designed and purchased from Ambion (Austin, TX), were tested for each of the two alpha subunit isoforms. The oligonucleotides with the highest knockdown efficiency for α1 and α2 were respectively: CGA GUU GAC UGG ACA UAA ATT (siRNA ID#: 194424) and GCA ACU AUC AAA GAC AUA CTT (siRNA ID#: 194794). As these cells express both alpha isoforms, the combination of the two nucleotides led to the greatest knockdown efficiency. Ambion's Negative Control #1 siRNA (Cat

#:4635) was used as a control in all experiments. For all knockdown experiments cells were seeded as described. Cells were first transfected at approximately 48 h post seeding (or ~60% confluency). A calcium phosphate transfection protocol was utilized as follows, 60 pmol of siRNA was added to siRNA mix: 15 µl ddH_2O, 15 µl Buffer A (0.5 M $CaCl_2$, 0.1 M HEPES (pH 7.0)) and 30 µl Buffer B (0.28 M NaCl, 0.75 mM NaH_2PO_4, 0.75 mM Na_2HPO_4 and 0.05 M HEPES (pH 7.0)) and after 10 min at room temperature, the siRNA mix was added to each well of a 12 well plate containing 600 µl DMEM + 5% FBS and incubated 12-16 h. Following 12-16 h incubation, media was aspirated and replaced with DMEM + 2% FBS. Additional transfection was repeated 72 h after initial transfection. Cells were treated and assayed 72 h after final transfection.

Statistics

All values are presented as means ±SEM. The significance of differences between means was evaluated by one-way repeated measures analysis of variance (ANOVA). Where differences among groups were indicated, the Newman-Keuls *post-hoc* test was used for post hoc comparison between groups. Differences between two groups were analyzed by the Student's two-tailed *t*-test for independent samples. GraphPad Prism 5 software was used for all statistical analyses. $P < 0.05$ was considered significant.

Primers used for PCR amplification in this study		
Name	**Primer Direction**	**Sequences (5'-3')**
pGL_2B-Hmgcr	F R	TAGGTACCCATCCCCTGTTCCCCGCG TAAAGCTTGTCTCCAGCCAACGGAGC
ChIP-Hmgcr	F R	ACCCGTCATTGGTTGGCTCT CTCCCTAACAACCGCCAACT
ChIP-Srebf1	F R	CCATCCCTGGCCCTTTAATCTAACGA TTCGGACTAGGCCCACGTTAAGGAAA
qPCR- Hmgcr	F R	TGTGGGAACGGTGACACTTA CTTCAAATTTTGGGCACTCA
qPCR-36B4	F R	AAGCGCGTCCTGGCATTGTCT CCGCAGGGGCAGCAGTGGT

Appendix A. Primers Utilized for PCR amplification in the current studies.

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Curriculum Vitae

Brent A. Penque

EDUCATION

Indiana University, Indianapolis, Indiana (IUPUI Campus)
August 2008-May 2013
Ph.D., Cellular and Integrative Physiology, Diabetes and Obesity minor
Thesis: Mechanisms of Hexosamine-Induced Cholesterol Accumulation and Therapeutic Actions of Chromium

Valparaiso University, Valparaiso, Indiana
August 2003-May 2007
B.S., Biology and Chemistry majors, Physics and Mathematics minors

FELLOWSHIPS

Indiana University School of Medicine
Moenkhaus Endowment for Academic Excellence
May 2009-May 2010

PUBLICATIONS

MANUSCRIPTS

Penque BA, Tackett L, Hoffman NJ, et al., *A potential mechanism for chromium action in the maintenance of glucose homeostasis*. *(In Preparation)*

Hoffman NJ, **Penque BA**, Habegger KM, et al., *Chromium enhances skeletal muscle insulin responsiveness via AMPK stimulation*. *(In Submission)*

Penque BA, Hoggatt AM, Herring BP, et al., *Hexosamine biosynthesis impairs insulin action via a cholesterolgenic response*. Molecular Endocrinology, 2013. **27**(3):p.536-47.

Habegger KM, **Penque BA**, Sealls W, et al., *Fat-induced membrane cholesterol accrual provokes cortical filamentous actin destabilisation and glucose transport dysfunction in skeletal muscle*. Diabetologia, 2012. **55**(2): p. 457-67.

Seals W, **Penque BA**, and Elmendorf JS, *Evidence that chromium modulates cellular cholesterol homeostasis and ABCA1 functionality impaired by hyperinsulinemia – brief report.* Atherioscler Thromb Vasc Biol, 2011. **31**(5): p.1139-40.

ABSTRACTS

Penque BA and Elmendorf JS, Hexosamines Provoke Membrane Cholesterol Accrual, Filamentous Actin Loss, and GLUT4 Dysregulation in Adipocytes through Transcriptional Activation of Specificity Protein 1. 2012 Keystone Symposium, Pathogenesis of Diabetes: Emerging Insights into Molecular Mechanisms

Penque BA, Sealls W, and Elmendorf JS, Evidence that Hyperinsulinemia, known to Accelerate Diabetes Progression, may also contribute to Dyslipidemia via Impairing ApoA1/ABCA1-Mediated Cholesterol Efflux. 2010, Diabetes, 59, Supplement 1

Sealls W, **Penque BA**, and Elmendorf JS, Insulin-Resistant Adipocytes Display Defective ABCA1/ApoA1-Mediated Cholesterol Efflux that AMPK Signaling Improves, Highlighting a Therapeutic Strategy to Raise High-Density Lipoprotein. 2010, Diabetes, 59, Supplement 1

PROFESSIONAL CONFERENCES ATTENDED

- 3rd Annual Indiana Physiological Society Meeting
  February 2013
  Indianapolis, IN
- 2012 Keystone Symposia Conference Series
  February 2012
  Pathogenesis of Diabetes: Emerging Insights into Molecular Mechanisms
  Santa Fe, NM
- 2nd Annual Indiana Physiological Society Meeting
  February 2012
  Muncie, IN
- 1st Annual Indiana Physiology Society Meeting
  February 2011
  Indianapolis, IN
- 70th Scientific Sessions of the American Diabetes Association
  June 2010
  Orlando, FL

PROFESSIONAL AFFILIATIONS

- Sigma Xi Society, Student Member
  2012-2013

- Indiana Physiology Society, Student Member
  2010-2013
- American Physiological Society, Student Member
  2009-2013
- Golden Key Honors Society
  2008-2013

PRESENTATIONS

- Research Poster Presentation
  February 2013
  3rd Annual Indiana Physiological Society Meeting
- Oral Presentation
  October 2012
  Indiana University Center for Diabetes Research Seminar Series
- Research Poster Presentation
  April 2012
  2012 IUPUI Research Day
- Research Poster Presentation
  February 2012
  Keystone Symposium: Pathogenesis of Diabetes
- Research Poster Presentation
  February 2012
  2nd Annual Indiana Physiological Society Meeting
- Oral Presentation
  January 2012
  Metabolism and Islet Biology Seminar Series
- Thesis Proposal Oral Presentation
  September 2011
  Department of Cellular & Integrative Physiology Seminar Series
- Oral Presentation
  August 2011
  2011 Sigma Xi Biomedical Research Competition
- Research Poster Presentation
  April 2011
  2011 IUPUI Research Day
- Research Poster Presentation
  February 2011
  Indiana Biomedical Gateway Program Recruitment Poster Session
- Research Poster Presentation
  February 2011
  1st Annual Indiana Physiological Society Meeting
- Oral Presentation
  January 2011
  IUPUI Center for Membrane Biosciences Meeting

- Oral Presentation
  August 2010
  Department of Cellular & Integrative Physiology Seminar Series
- Research Poster Presentation
  June 2010
  70th Scientific Sessions of the American Diabetes Association
- Oral Presentation
  May 2010
  Sigma Xi Biomedical Research Competition
- Research Poster Presentation
  October 2009
  2009 Indiana Physiology Statewide Departmental Retreat

AWARDS

- National Institute of Diabetes
  2012
  Travel Award for Keystone Symposia
- Graduate and Professional Student Government
  2011
  Educational Enhancement Grant
- 1st Place Presentation: 2011 Sigma Xi Biomedical Research Competition
  2011
- 3rd Place Presentation: 2010 Sigma Xi Biomedical Research Competition
  2010
- Indiana University Travel Fellowship
  2008
- Induction into Golden Key Honor Society
  2008
- Induction into Phi Lambda Upsilon Chemistry Honor Society
  2007
- Mini Marathon Finisher
  2007
- Valparaiso University Deans Award
  2003-2007

RESEARCH INTERESTS

- Cellular and molecular mechanisms of insulin resistance
- Regulation of glucose and lipid metabolism
- Mechanisms of lipoprotein metabolism

RESEARCH EXPERTISE

- Cell culture and treatments (L6 myotubes and 3T3-L1 adipocytes)
- Preparation and analysis of protein: SDS-PAGE
- Western blot analyses
- Whole cell immunofluorescence
- Plasma membrane sheet assays
- Subcellular fractionation
- Fluorescent and confocal microscopy in cultured cells and intact tissue
- Animal care and maintenance (mice)
- Dissection: rodent brain, heart, liver, kidney, pancreas, hindlimb and soleus muscle, epididymal fat pads
- Intraperitoneal glucose tolerance testing (mice)
- Intraperitoneal insulin tolerance testing (mice)
- Blood collection (mice and rats)
- Enzyme-linked immunosorbent assays
- 2-deoxyglucose uptake assays (skeletal muscle tissue and cultured cells)
- Amplex red cholesterol assay
- Immunoprecipitation
- Chromatin immunoprecipitation
- Primer design
- Electroporation and plasmid transfection
- Luciferase assays
- siRNA knockdown
- Preparation and analysis of DNA and RNA: PCR, sequencing, agarose and non-denaturing polyacrylamide gel electrophoresis, plasmid isolation, restriction digest, ligation, vector construction, isolation and purification of DNA and RNA

TRAINING EXPERIENCES

Indiana University School of Medicine
May 2009-May 2013
Department of Cellular and Integrative Physiology
Mentor: Jeffrey S. Elmendorf, Ph.D.

Indiana University School of Medicine
January 2009-May 2009
Department of Cellular & Integrative Physiology
Mentor: David P. Basile, Ph.D.

Indiana University School of Medicine
August 2008-January 2009
Department of Neuroscience
Mentor: Dena Davidson, Ph.D.

Valparaiso University
August 2006-May 2007
Developmental Biology Department
Mentor: Grayson Davis, Ph.D.

Purdue University
May 2006-August 2006
Birck Nanotechnology Center, Electrical and Computer Engineering Department
Mentor: David Janes, Ph.D.

TEACHING EXPERIENCE

Indiana University School of Medicine
G715 Biochemical Basis of Biological Processes
August 2010-May 2011
Teaching Assistant

Valparaiso University: Department of Biology
BIO-171 and BIO-172 Unity and Diversity of Life
August 2005-May 2007
Laboratory Assistant

Valparaiso University: Department of Physics
PHYS-141 and PHYS-142 Mechanics and Heat, Electricity Magnetism & Waves
August 2006-May 2007
Teaching Assistant

PROFESSIONAL AND UNIVERSITY SERVICE

- Indiana University School of Medicine Student Mentor
  2012-2013
- Graduate and Professional Student Government
  2011-2013
  President, School of Medicine
- Indiana Physiological Society
  2010-2013
  Student Councilor
- Indiana University School of Medicine Graduate Committee
  2009-2013
  Student Representative
- Indiana University School of Medicine Student Ambassador
  2009-2013
  Student Coordinator for Campus Visits
- Indiana University School of Medicine Campus Visit Student Panel
  2012
  Member and Mock Interviewee

- Indiana University School of Medicine Getting You into IUPUI
  2012
  Student Panel Member
- Department of Cellular & Integrative Physiology Retreat
  2011
  Moderator and Organizer for Graduate Student Session
- Valparaiso University: Residential Life
  2005-2006
  Resident Assistant

COMMUNITY SERVICE

- Valparaiso University
  2006-2007
  Judicial Board Coordinator
- Valparaiso University
  2006-2007
  Biology Club Vice President
- Valparaiso University
  2006-2007
  Habitat for Humanity Build Coordinator
- Valparaiso University Café Manna
  2005-2007
  Student Volunteer, Prepared & Served Meals to Underprivileged Families
- Campus Crusade for Christ
  2004-2007
  Leadership Team, Outreach Coordinator